



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/AU98/00246 <b>(22) International Filing Date:</b> 9 April 1998 (09.04.98)  <b>(30) Priority Data:</b> <table border="0" style="width: 100%;"> <tr> <td style="width: 30%;">PO 6223</td> <td style="width: 40%;">15 April 1997 (15.04.97)</td> <td style="width: 30%;">AU</td> </tr> <tr> <td>PO 6226</td> <td>15 April 1997 (15.04.97)</td> <td>AU</td> </tr> <tr> <td>60/043,706</td> <td>16 April 1997 (16.04.97)</td> <td>US</td> </tr> <tr> <td>60/050,403</td> <td>20 June 1997 (20.06.97)</td> <td>US</td> </tr> </table> <b>(71) Applicant (for all designated States except US):</b> COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU).  <b>(71)(72) Applicant and Inventor:</b> STYMNE, Sten [SE/SE]; PI 1380, S-268 90 Svalov (SE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> GREEN, Allan [AU/AU]; 5 Barton Court, Bourke Street, Barton, ACT 2601 (AU). SINGH, Surinder [AU/AU]; 10 Lucas Place, Downer, ACT 2602 (AU). LENMAN, Marit [SE/SE]; Revingegatan 13a, S-223 56 Lund (SE).		PO 6223	15 April 1997 (15.04.97)	AU	PO 6226	15 April 1997 (15.04.97)	AU	60/043,706	16 April 1997 (16.04.97)	US	60/050,403	20 June 1997 (20.06.97)	US	<b>(74) Agents:</b> SLATTERY, John, Michael et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
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<b>(54) Title:</b> PLANT FATTY ACID EPOXYGENASE GENES AND USES THEREFOR  <b>(57) Abstract</b> <p>The present invention relates generally to novel genetic sequences which encode fatty acid epoxxygenase enzymes. In particular, the present invention relates to genetic sequences which encode fatty acid <math>\Delta 12</math>-epoxxygenase enzymes comprising mixed function monooxygenase enzymes. More preferably, the present invention provides cDNA sequences which encode plant fatty acid epoxxygenases, in particular the <i>Crepis palaestina</i> <math>\Delta 12</math>-epoxxygenase and homologues, analogues and derivatives thereof. The genetic sequences of the present invention provide the means by which fatty acid metabolism may be altered or manipulated in organisms such as yeasts, moulds, bacteria, insects, birds, mammals and plants, in particular to convert unsaturated fatty acids to epoxxygenated fatty acids therein. The invention extends to genetically modified oil-accumulating organisms transformed with the subject genetic sequences and to the oils derived therefrom. The oils thus produced provide the means for the cost-effective raw materials for use in the efficient production of coatings, resins, glues, plastics, surfactants and lubricants, amongst others.</p>														

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## PLANT FATTY ACID EPOXYGENASE GENES AND USES THEREFOR

### FIELD OF THE INVENTION

The present invention relates generally to novel genetic sequences which encode fatty acid epoxygenase enzymes. In particular, the present invention relates to genetic sequences which encode fatty acid  $\Delta$ 12-epoxygenase enzymes as defined herein. More particularly, the present invention provides cDNA and genomic gene sequences which encode plant fatty acid epoxygenases, preferably *Crepis palaestina* or *Euphorbia lagascae*  $\Delta$ 12-epoxygenases. The genetic sequences of the present invention provide the means by which fatty acid metabolism may be altered or manipulated in organisms such as yeasts, moulds, bacteria, insects, birds, mammals and plants, in particular to convert unsaturated fatty acids to epoxygenated fatty acids therein. The invention extends to genetically modified oil-accumulating organisms transformed with the subject genetic sequences and to the oils derived therefrom. The oils thus produced provide the means for the cost-effective raw materials for use in the efficient production of coatings, resins, glues, plastics, surfactants and lubricants, amongst others.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Sequence identity numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined after the bibliography.

### BACKGROUND TO THE INVENTION

There is considerable interest world-wide in producing chemical feedstock, such as fatty acids, for industrial use from renewable plant sources rather than from non-renewable petrochemicals. This concept has broad appeal to manufacturers and consumers on the basis

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of resource conservation and provides a significant opportunity to develop new industrial crops for agriculture.

There is a diverse array of unusual fatty acids in nature and these have been well  
5 characterised (Badam & Patil, 1981; Smith, 1970). Many of these unusual fatty acids have industrial potential and this has led to interest in domesticating such species to enable agricultural production of particular fatty acids.

One class of fatty acids of particular interest are the epoxy-fatty acids, consisting of  
10 an acyl chain in which two adjacent carbon bonds are linked by an epoxy bridge. Due to their high reactivities, they have considerable application in the production of coatings, resins, glues, plastics, surfactants and lubricants. These fatty acids are currently produced by chemical epoxidation of vegetable oils, mainly soybean oil and linseed oil, however this process produces mixtures of multiple and isomeric forms and involves significant processing  
15 costs.

Attempts are being made by others to develop some wild plants that contain epoxy fatty acids (eg. *Euphorbia lagascae*, *Vernonia galamensis*) into commercial sources of these oils. However, problems with agronomic suitability and low yield potential severely limit  
20 the commercial utility of traditional plant breeding and cultivation approaches.

The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating the efficiency of commercially-important industrial processes, by the expression of genes isolated from a first organism or species in a second organism or species to confer  
25 novel phenotypes thereon. More particularly, conventional industrial processes can be made more efficient or cost-effective, resulting in greater yields per unit cost by the application of recombinant DNA techniques.

Moreover, the appropriate choice of host organism for the expression of a genetic  
30 sequence of interest provides for the production of compounds which are not normally

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produced or synthesized by the host, at a high yield and purity.

However, despite the general effectiveness of recombinant DNA technology, the isolation of genetic sequences which encode important enzymes in fatty acid metabolism, in particular the genes which encode the fatty acid  $\Delta 12$ -epoxygenase enzymes responsible for producing 12,13-epoxy-9-octadecenoic acid (vernolic acid) and 12,13-epoxy-9,15-octadecadienoic acid, amongst others, remains a major obstacle to the development of genetically-engineered organisms which produce these fatty acids.

Until the present invention, there were only limited biochemical data indicating the nature of fatty acid epoxygenase enzymes, in particular  $\Delta 12$ -epoxygenases. However, in *Euphorbia lagascae*, the formation of 12,13-epoxy-9-octadecenoic acid (vernolic acid) from linoleic acid appears to be catalysed by a cytochrome-P450-dependent  $\Delta 12$  epoxygenase enzyme (Bafor *et al.*, 1993; Blee *et al.*, 1994). Additionally, developing seed of linseed plants have the capability to convert added vernolic acid to 12,13-epoxy-9,15-octadecadienoic acid by an endogenous  $\Delta 15$  desaturase (Engeseth and Stymne, 1996). Epoxy-fatty acids can also be produced by a peroxide-dependent peroxygenase in plant tissues (Blee and Schubert, 1990).

In work leading up to the present invention, the inventors sought to isolate genetic sequences which encode genes which are important for the production of epoxy-fatty acids, such as 12,13-epoxy-9-octadecenoic acid (vernolic acid) or 12,13-epoxy-9,15-octadecadienoic acid and to transfer these genetic sequences into highly productive commercial oilseed plants and/or other oil accumulating organisms.

## SUMMARY OF THE INVENTION

One aspect of the invention provides an isolated nucleic acid molecule which encodes or is complementary to an isolated nucleic acid molecule which encodes a fatty acid epoxygenase.

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A second aspect of the invention provides an isolated nucleic acid molecule which hybridizes under at least low stringency conditions to at least 20 contiguous nucleotides of SEQ ID NOs:1 or 3 or 5 or 19 or 20, or a complementary sequence thereto.

5 A further aspect of the invention provides isolated nucleic acid molecule which comprises a sequence of nucleotides which is at least 65% identical to SEQ ID NO:1 or 3 or 5 or which is at least 75% identical to at least 200 contiguous nucleotides in SEQ ID NOs: 19 or 20, or a complementary sequence thereto.

10 A further aspect of the invention provides a genetic construct which comprises the isolated nucleic acid molecule *supra*, in either the sense or antisense orientation, in operable connection with a promoter sequence.

A further aspect of the invention provides a method of altering the level of epoxy fatty  
15 acids in a cell, tissue, organ or organism, said method comprising expressing a sense, antisense, ribozyme or co-suppression molecule comprising the isolated nucleic acid molecule *supra* in said cell for a time and under conditions sufficient for the level of epoxy fatty acids therein to be increased or reduced.

20 A further aspect of the invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising expressing the isolated nucleic acid molecule *supra* in said cell for a time and under conditions sufficient for the epoxygenase encoded therefor to be produced.

25 A further aspect of the invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising the steps of:

(i) producing a genetic construct which comprises the isolated nucleic acid molecule *supra* placed operably under the control of a promoter capable of conferring  
30 expression on said genetic sequence in said cell, and optionally an expression

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enhancer element;

- (ii) transforming said genetic construct into said cell; and
- (iii) selecting transformants which express a functional epoxygenase encoded by the genetic sequence at a high level.

5

A still further aspect of the invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a transgenic plant comprising the steps of:

- (i) producing a genetic construct which comprises the isolated nucleic acid molecule *supra* placed operably under the control of a seed-specific promoter and optionally an expression enhancer element, wherein said genetic sequences is also placed upstream of a transcription terminator sequence;
- (ii) transforming said genetic construct into a cell or tissue of said plant; and
- (iii) selecting transformants which express a functional epoxygenase encoded by the genetic sequence at a high level in seeds.

15

A further aspect of the invention provides a recombinant epoxygenase polypeptide or functional enzyme molecule.

- 20 A further aspect of the invention provides a recombinant epoxygenase which comprises a sequence of amino acids set forth in any one of SEQ ID NOs: 2 or 4 or 6 or a homologue, analogue or derivative thereof which is at least about 50% identical thereto.

A still further aspect of the invention provides a method of producing an epoxygenated fatty acid in a cell, tissue, organ or organism, said method comprising incubating a cell, tissue, organ or organism which expresses an enzymatically active recombinant epoxygenase with a fatty acid substrate and preferably, an unsaturated fatty acid substrate, for a time and under conditions sufficient for at least one carbon bond, preferably a carbon double bond, of said substrate to be converted to an epoxy group.

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A further aspect of the invention provides an immunologically interactive molecule which binds to the recombinant epoxygenase polypeptide described herein or a homologue, analogue or derivative thereof.

## 5 BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** is a linear representation of an expression plasmid comprising an epoxygenase structural gene, placed operably under the control of the truncated napin promoter (FP1; right-hand hatched box) and placed upstream of the NOS terminator sequence (right-hand  
10 stippled box). The epoxygenase genetic sequence is indicated by the right-hand open rectangular box. The construct also comprises the NOS promoter (left-hand hatched box) driving expression of the *NPTII* gene (left-hand open box) and placed upstream of the NOS terminator (left-hand stippled box). The left and right border sequences of the *Agrobacterium tumefaciens* Ti plasmid are also indicated.

15

**Figure 2** is a schematic representation showing the alignment of the amino acid sequences of the epoxygenase polypeptide of *Crepis palaestina* (Cpa12; SEQ ID NO:2), a further epoxygenase derived from *Crepis sp.* other than *C. palaestina* which produces high levels of vernolic acid (CrepX; SEQ ID NO:4), a partial amino acid sequence of an epoxygenase  
20 polypeptide derived from *Vernonia galamensis* (Vgal1; SEQ ID NO:6), the amino acid sequence of the  $\Delta 12$  acetylenase of *Crepis alpina* (Crep1; SEQ ID NO:8), the  $\Delta 12$  desaturases of *A. thaliana* (L26296; SEQ ID NO:9), *Brassica juncea* (X91139; SEQ ID NO:10), *Glycine max* (L43921; SEQ ID NO:11), *Solanum commersonii* (X92847; SEQ ID NO:12) and *Glycine max* (L43920; SEQ ID NO:13), and the  $\Delta 12$  hydroxylase of *Ricinus*  
25 *communis* (U22378; SEQ ID NO:14). Underlined are three histidine-rich motifs that are conserved in non-heme containing mixed-function monooxygenases.

**Figure 3** is a copy of a photographic representation of a northern blot hybridization showing seed-specific expression of the *Crepis palaestina* epoxygenase gene exemplified by SEQ ID  
30 NO:1. Northern blot analysis of total RNA from leaves (lane 1) and developing seeds (lane

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2) of *Crepis palaestina*. 15 µg of total RNA was run on a Northern gel and blotted onto Hybond N<sup>+</sup> membrane from Amersham according to the manufacturer's instructions. The blot was hybridized at 60°C with a probe made from the 3' untranslated region of SEQ ID NO: 1. The blot was washed twice in 2 x SSC (NaCl- Sodium Citrate buffer) at room temperature for 10 minutes, then in 0.1xSSC at 60°C for 20 min.

**Figure 4** is a schematic representation showing the nucleotide sequence of the degenerate PCR primer (5' to 3' direction) used to isolate the *Euphorbia lagascae* epoxygenase genes described herein.

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**Figure 5** is a copy of a photographic representation of a RNA dot blot hybridization showing expression of the epoxygenase gene exemplified in SEQ ID NO:3 in plants which produce vernolic acid compared to plants which do not produce vernolic acid. One µg of total RNA was isolated from the specified tissue and dot blotted onto the Hybond N<sup>+</sup> membrane from Amersham as per the manufacturer's instructions. The blot was hybridised at 42°C in 50% formamide with the relevant <sup>32</sup>P labelled probe made from SEQ ID NO: 3 for 16 hours. Blots were washed twice in 2x SSC (NaCl- Sodium Citrate buffer) at room temperature then in 0.5x SSC at 55°C for 20 minutes. Autoradiographs were obtained after an overnight exposure. Panel A shows total RNA from developing seed of *Euphorbia lagascae* (1), *Euphorbia cyparissus* (2), *Vernonia galamensis* (3), and flax (*Linum usitatissimum*)(4). Panel B shows total RNA from various tissues of *Euphorbia lagascae*, including developing seed (1), root (2) and leaf (3).

**Figure 6** is a schematic representation showing the subtractive hybridization method used to isolate the *Euphorbia lagascae* epoxygenase genes described herein. The +6cDNA pool consisted predominantly of seed storage protein-like sequences. A pool of 15 such sequences were biotinylated and further subtracted from the +6cDNA. LH = Long Hybridisation - 20 hrs; SH = Short Hybridisation - 3 hrs.

**Figure 7** is a copy of a photographic representation of a RNA dot blot hybridization showing

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expression of the epoxigenase gene exemplified in SEQ ID NO:20 in plants which produce vernolic acid compared to plants which do not produce vernolic acid. One  $\mu\text{g}$  of total RNA was isolated from the specified tissue and dot blotted onto the Hybond N<sup>+</sup> membrane from Amersham as per the manufacturer's instructions. The blot was hybridised at 42°C in 50% formamide with the relevant <sup>32</sup>P labelled probe made from SEQ ID NO:20 for 16 hours. Blots were washed twice in 2x SSC (NaCl- Sodium Citrate buffer) at room temperature then in 0.5x SSC at 55°C for 20 minutes. Autoradiographs were obtained after an overnight exposure. Panel A shows total RNA from developing seed of *Euphorbia lagascae* (1), *Euphorbia cyparissus* (2), *Vernonia galamensis* (3) and flax (*Linum usitatissimum*) (4). Panel B shows total RNA from various tissue of *Euphorbia lagascae*, including developing seed (1), root (2) and leaf (3).

Figure 8 is a schematic representation of a binary plasmid vector containing an expression cassette which comprises the truncated napin seed-specific promoter (Napin) and nopaline synthase terminator (NT), with a *Bam*HI cloning site there between, in addition to the kanamycin-resistance gene *NPTII* operably connected to the nopaline synthase promoter (NP) and nopaline synthase terminator (NT) sequences. The expression cassette is flanked by T-DNA left border (LB) and right-border (RB) sequences.

Figure 9 is a schematic representation of a binary plasmid vector containing an expression cassette which comprises SEQ ID NO: 1 placed operably under the control of a truncated napin seed-specific promoter (Napin) and upstream of the nopaline synthase terminator (NT), in addition to the kanamycin-resistance gene *NPTII* operably connected to the nopaline synthase promoter (NP) and nopaline synthase terminator (NT) sequences. The expression cassette is flanked by T-DNA left border (LB) and right-border (RB) sequences. To produce this construct, SEQ ID NO:1 is inserted into the *Bam*HI site of the binary vector set forth in Figure 8.

Figure 10 is a graphical representation of gas-chromatography traces of fatty acid methyl esters prepared from oil seeds of untransformed *Arabidopsis thaliana* plants [panel (a)], or

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*A. thaliana* plants (transgenic line Cpal-17) which have been transformed with SEQ ID NO:1 using the genetic construct set forth in Figure 9 [panels (b) and (c)]. In panels (a) and (b), fatty acid methyl esters were separated using packed column separation. In panel (c), the fatty acid methyl esters were separated using capillary column separation. The elution positions of vernolic acid are indicated.

**Figure 11** is a graphical representation showing the joint distribution of epoxy fatty acids in selfed seed on T<sub>1</sub> plants of Cpal2-transformed *Arabidopsis thaliana* plants as determined using gas chromatography. Levels of both vernolic acid (x-axis) and 12,13-epoxy-9,15-octadecadienoic acid (y-axis) were determined and plotted relative to each other. Data show a positive correlation between the levels of these fatty acids in transgenic plants.

**Figure 12** is a graphical representation showing the incorporation of <sup>14</sup>C-label into the chloroform phase obtained from lipid extraction of linseed cotyledons during labelled-substrate feeding. Symbols used; ♦, [<sup>14</sup>C]oleic acid feeding; ■, [<sup>14</sup>C]vernolic acid feeding.

**Figure 13** is a graphical representation showing the incorporation of <sup>14</sup>C-label into the phosphatidylcholine of linseed cotyledons during labelled-substrate feeding. Symbols used; ♦, [<sup>14</sup>C]oleic acid feeding; ■, [<sup>14</sup>C]vernolic acid feeding.

**Figure 14** is a graphical representation showing the incorporation of <sup>14</sup>C-label into the triacylglycerols of linseed cotyledons during labelled-substrate feeding. Symbols used; ♦, [<sup>14</sup>C]oleic acid feeding; ■, [<sup>14</sup>C]vernolic acid feeding.

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**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

One aspect of the present invention provides an isolated nucleic acid molecule which encodes or is complementary to an isolated nucleic acid molecule which encodes a fatty acid  
5 epoxygenase.

Wherein the isolated nucleic acid molecule of the invention encodes an enzyme which is involved in the direct epoxidation of arachidonic acid, it is particularly preferred that the subject nucleic acid molecule is derived from a non-mammalian source.

10

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

15 The term "non-mammalian source" refers to any organism other than a mammal or a tissue or cell derived from same.

In the present context, the term "derived from a non-mammalian source" shall be taken to indicate that a particular integer or group of integers has been derived from bacteria,  
20 yeasts, birds, amphibians, reptiles, insects, plants, fungi, moulds and algae or other non-mammal.

In a preferred embodiment of the present invention, the source organism is any such organism possessing the genetic capacity to synthesize epoxy fatty acids. More preferably,  
25 the source organism is a plant such as, but not limited to *Chrysanthemum spp.*, *Crepis spp.*, *Euphorbia spp.* and *Vernonia spp.*, amongst others.

Even more preferably, the source organism is selected from the list comprising *Crepis biennis*, *Crepis aurea*, *Crepis conyzaefolia*, *Crepis intermedia*, *Crepis occidentalis*, *Crepis*  
30 *palaestina*, *Crepis vesicaria*, *Crepis xacintha*, *Euphorbia lagascae* and *Vernonia galamensis*.

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Additional species are not excluded.

In a particularly preferred embodiment of the present invention, the source organism is a *Crepis sp.* which contains high levels of vernolic acid such as *Crepis palaestina*, amongst 5 others or alternatively, *Vernonia galamensis* or *Euphorbia lagascae*.

Wherein the isolated nucleic acid molecule of the invention encodes a  $\Delta 6$ -epoxygenase or  $\Delta 9$ -epoxygenase enzyme or  $\Delta 12$ -epoxygenase or  $\Delta 15$ -epoxygenase enzyme, or at least encodes an enzyme which is not involved in the direct epoxidation of arachidonic acid, the 10 subject nucleic acid molecule may be derived from any source producing said enzyme, including, but not limited to, yeasts, moulds, bacteria, insects, birds, mammals and plants.

The nucleic acid molecule of the invention according to any of the foregoing embodiments may be DNA, such as a gene, cDNA molecule, RNA molecule or a synthetic 15 oligonucleotide molecule, whether single-stranded or double-stranded and irrespective of any secondary structure characteristics unless specifically stated.

Reference herein to a "gene" is to be taken in its broadest context and includes:

- (i) a classical genomic gene consisting of transcriptional and/or translational regulatory 20 sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences); or
- (ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene.

25 The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product. Preferred epoxygenase genes of the present invention may be derived from a naturally-occurring epoxygenase gene by standard recombinant techniques. Generally, an epoxygenase gene may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions.

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Nucleotide insertional derivatives include 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotides are introduced into a predetermined site in the nucleotide sequence although random insertion is also possible with suitable screening of the  
5 resulting product.

Deletional variants are characterised by the removal of one or more nucleotides from the sequence.

10 Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon. Alternatively, substituents are designed to alter one amino acid for another similar acting amino acid, or amino acid of like charge, polarity, or hydrophobicity.

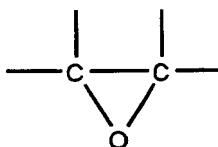
15 In the context of the present invention, the term "fatty acid epoxygenase" shall be taken to refer to any enzyme or functional equivalent or enzymatically-active derivative thereof which catalyzes the biosynthesis of an epoxygenated fatty acid, by converting a carbon bond of a fatty acid to an epoxy group and preferably, by converting a carbon double  
20 bond of an unsaturated fatty acid to an epoxy group. Although not limiting the invention, a fatty acid epoxygenase may catalyze the biosynthesis of an epoxy fatty acid selected from the list comprising 12,13-epoxy-9-octadecenoic acid (vernolic acid), 12,13-epoxy-9,15-octadecadienoic acid, 15,16-epoxy-9,12-octadecadienoic acid, 9,10-epoxy-12-octadecenoic acid, and 9,10-epoxy-octadecanoic acid, amongst others.

25 The term "epoxy", "epoxy group" and "epoxy residue" will be known by those skilled in the art to refer to a three membered ring comprising two carbon atoms and an oxygen atom linked by single bonds as follows:

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Accordingly, the term "epoxide" refers to compounds which comprise at least one epoxy group as hereinbefore defined.

- 10 Those skilled in the art are aware that fatty acid nomenclature is based upon the length of the carbon chain and the position of unsaturated carbon atoms within that carbon chain. Thus, fatty acids are designated using the shorthand notation:

Carbon<sub>total</sub> : double bond<sub>total</sub> double bond( $\Delta$ ) position,

15

wherein the double bonds are *cis* unless otherwise indicated. For example, palmitic acid (*n*-hexadecanoic acid) is a saturated 16-carbon fatty acid (i.e. 16:0), oleic acid (octadecenoic acid) is an unsaturated 18-carbon fatty acid with one double bond between C-9 and C-10 (i.e. 18:1 <sup>$\Delta^9$</sup> ), and linoleic acid (octadecadienoic acid) is an unsaturated 18-carbon fatty acid with  
20 two double bonds between C-9 and C-10 and between C-12 and C-13 (i.e. 18:2 <sup>$\Delta^{9,12}$</sup> ).

However, in the present context an epoxxygenase enzyme may catalyze the conversion of any carbon bond to an epoxy group or alternatively, the conversion of any double in an unsaturated fatty acid substrate to an epoxy group. In this regard, it is well-known by those  
25 skilled in the art that most mono-unsaturated fatty acids of higher organisms are 18-carbon unsaturated fatty acids (i.e. 18:1  <sup>$\Delta^9$</sup> ), while most polyunsaturated fatty acids derived from higher organisms are 18-carbon fatty acids with at least one of the double bonds therein located between C-9 and C-10. Additionally, bacteria also possess C16- mono-unsaturated fatty acids. Moreover, the epoxxygenase of the present invention may act on more than a  
30 single fatty acid substrate molecule and, as a consequence, the present invention is not to be

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limited by the nature of the substrate molecule upon which the subject epoxygenase enzyme acts.

Preferably, the substrate molecule for the epoxygenase of the present invention is an  
5 unsaturated fatty acid which contains at least one double bond.

Furthermore, epoxygenase enzymes may act upon any number of carbon atoms in any one substrate molecule. For example, they may be characterised as  $\Delta 6$ -epoxygenase,  $\Delta 9$ -epoxygenase,  $\Delta 12$ -epoxygenase or  $\Delta 15$ -epoxygenase enzymes amongst others. Accordingly,  
10 the present invention is not limited by the position of the carbon atom in the substrate upon which an epoxygenase enzyme may act.

The term " $\Delta 6$ -epoxygenase" as used herein shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the  $\Delta 6$  carbon bond of a fatty acid substrate to a  
15  $\Delta 6$  epoxy group and preferably, catalyzes the conversion of the  $\Delta 6$  double bond of at least one unsaturated fatty acid to a  $\Delta 6$  epoxy group.

The term " $\Delta 9$ -epoxygenase" as used herein shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the  $\Delta 9$  carbon bond of a fatty acid substrate to a  
20  $\Delta 9$  epoxy group and preferably, catalyzes the conversion of the  $\Delta 9$  double bond of at least one unsaturated fatty acid to a  $\Delta 9$  epoxy group.

As used herein, the term " $\Delta 12$ -epoxygenase" shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the  $\Delta 12$  carbon bond of a fatty acid substrate to a  
25  $\Delta 12$  epoxy group and preferably, catalyzes the conversion of the  $\Delta 12$  double bond of at least one unsaturated fatty acid to a  $\Delta 12$  epoxy group.

As used herein, the term " $\Delta 15$ -epoxygenase" shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the  $\Delta 15$  carbon bond of a fatty acid substrate to a  
30  $\Delta 15$  epoxy group and preferably, catalyzes the conversion of the  $\Delta 15$  double bond of at least

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one unsaturated fatty acid to a  $\Delta 15$  epoxy group.

The present invention clearly extends to genetic sequences which encode all of the epoxygenase enzymes *listed supra*, amongst others.

5

In one preferred embodiment of the invention, the isolated nucleic acid molecule encodes a fatty acid epoxygenase enzyme which converts at least one carbon bond in palmitoleic acid (16:1  $\Delta^9$ ), oleic acid (18:1  $\Delta^9$ ), linoleic acid (18:2  $\Delta^{9,12}$ ), linolenic acid (18:3  $\Delta^{9,12,15}$ ), or arachidonic acid (20:4  $\Delta^{5,8,11,14}$ ) to an epoxy bond. Preferably, the carbon  
10 bond is a carbon double bond.

More preferably, the isolated nucleic acid molecule of the invention encodes a fatty acid epoxygenase enzyme which at least converts one or both double bonds in linoleic acid to an epoxy group. According to this embodiment, an epoxygenase which converts both the  
15  $\Delta^9$  and the  $\Delta^{12}$  double bonds of linoleic acid to an epoxy group may catalyze such conversions independently of each other such that said epoxygenase is a  $\Delta^9$ -epoxygenase and/or a  $\Delta^{12}$ -epoxygenase enzyme as hereinbefore defined.

In an alternative preferred embodiment, the fatty acid epoxygenase of the present  
20 invention is a  $\Delta^{12}$ -epoxygenase, a  $\Delta^{15}$ -epoxygenase or a  $\Delta^9$ -epoxygenase as hereinbefore defined.

More preferably, the fatty acid epoxygenase of the invention is a  $\Delta^{12}$ -epoxygenase as hereinbefore defined.

25

In a particularly preferred embodiment of the invention, there is provided an isolated nucleic acid molecule which encodes linoleate  $\Delta^{12}$ -epoxygenase, the enzyme which at least converts the  $\Delta^{12}$  double bond of linoleic acid to a  $\Delta^{12}$ -epoxy group, thereby producing 12,13-epoxy-9-octadecenoic acid (vernolic acid).

30

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Although not limiting the present invention, the preferred source of the  $\Delta 12$ -epoxygenase of the invention is a plant, in particular *Crepis palaestina* or a further *Crepis sp.* which is distinct from *C. palaestina* but contains high levels of vernolic acid, *Vernonia galamensis* or *Euphorbia lagascae*.

5

According to this embodiment, a  $\Delta 12$ -epoxygenase may catalyze the conversion of palmitoleic acid to 9,10-epoxy-palmitic acid and/or the conversion of oleic acid to 9,10-epoxy-stearic acid and/or the conversion of linoleic acid to any one or more of 9,10-epoxy-12-octadecenoic acid or 12,13-epoxy-9-octadecenoic acid or 9,10,12,13-diepoxy-stearic acid  
10 and/or the conversion of linolenic acid to any one or more of 9,10-epoxy-12,15-octadecadienoic acid or 12,13-epoxy-9,15-octadecadienoic acid or 15,16-epoxy-octadecadienoic acid or 9,10,12,13-diepoxy-15-octadecenoic acid or 9,10,15,16-diepoxy-12-octadecenoic acid or 12,13,15,16-diepoxy-9-octadecenoic acid or 9,10,12,13,15,16-triepoxy-stearic acid and/or the conversion of arachidonic acid to any one or more of 5,6-epoxy-  
15 8,11,14-tetracosatrienoic acid or 8,9-epoxy-5,11,14-tetracosatrienoic acid or 11,12-epoxy-5,8,14-tetracosatrienoic acid or 14,15-epoxy-5,8,11-tetracosatrienoic acid or 5,6,8,9-diepoxy-11,14-tetracosadienoic acid or 5,6,11,12-diepoxy-8,14-tetracosadienoic acid or 5,6,14,15-diepoxy-8,11-tetracosadienoic acid or 8,9,11,12-diepoxy-5,14-tetracosadienoic acid or 8,9,14,15-diepoxy-5,11-tetracosadienoic acid or 11,12,14,15-diepoxy-5,8-tetracosadienoic  
20 acid or 5,6,8,9,11,12-triepoxy-14-tetracosenoic acid or 5,6,8,9,14,15-triepoxy-11-tetracosenoic acid or 5,6,11,12,14,15-triepoxy-8-tetracosenoic acid or 8,9,11,12,14,15-triepoxy-5-tetracosenoic acid, amongst others.

Those skilled in the art may be aware that not all substrates listed *supra* may be  
25 derivable from a natural source, but notwithstanding this, may be produced by chemical synthetic means. The conversion of both naturally-occurring and chemically-synthesized unsaturated fatty acids to epoxy fatty acids is within the scope of the present invention, the only requirement being that the nucleic acid molecule of the present invention as described herein encodes an enzyme or functional part thereof which is capable of catalyzing said  
30 conversion.

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According to the preceding discussion, those skilled in the art will be aware that a fatty acid epoxxygenase may be a cytochrome-P450-dependent monooxygenase enzyme or a mixed-function monooxygenase enzyme or alternatively a peroxide-dependent peroxygenase enzyme, or like enzyme, amongst others. However, the present invention is particularly  
5 directed to those epoxxygenase enzymes which are mixed-function monooxygenase enzymes and nucleic acid molecules encoding same and uses therefor. Accordingly, it is particularly preferred that the nucleic acid molecule of the invention encode a fatty acid epoxxygenase which is a mixed-function monooxygenase enzyme.

10 In the context of the present invention, the term "mixed-function monooxygenase enzyme" shall be taken to refer to any enzyme which catalyzes the epoxxygenation of a carbon bond or carbon double bond in a fatty acid molecule, wherein said enzyme further comprises a sequence of amino acids which contains three histidine-rich regions as follows:

- 15 (i) His-(Xaa)<sub>3-4</sub>-His;  
(ii) His-(Xaa)<sub>2-3</sub>-His-His; and  
(iii) His-(Xaa)<sub>2-3</sub>-His-His,

wherein His designates histidine, Xaa designates any naturally-occurring amino acid residue  
20 as set forth in Table 1 herein, the integer (Xaa)<sub>3-4</sub> refers to a sequence of amino acids comprising three or four repeats of Xaa, and the integer (Xaa)<sub>2-3</sub> refers to a sequence of amino acids comprising two or three repeats of Xaa.

The term "mixed-function monooxygenase enzyme-like" shall be taken to refer to any  
25 enzyme which comprises three of the histidine-rich regions listed *supra*.

In the exemplification of the invention described herein, the inventors have demonstrated that the *Crepis palaestina* amino acid sequence provided herein comprises a  $\Delta 12$ -epoxxygenase enzyme which includes the characteristic amino acid sequence motifs of a mixed-  
30 function monooxygenase enzyme as hereinbefore defined. Close amino acid sequence identity

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between the *C. palaestina*  $\Delta$ 12-epoxygenase enzyme (SEQ ID NO: 2) and the amino acid sequences of polypeptides derived from an unidentified *Crepis sp.* and *Vernonia galamensis* as provided herein (SEQ ID NOs: 4 and 6), compared to the amino acid sequences of other mixed function monooxygenases such as desaturases and hydroxylases, suggests that said

5 *Crepis sp.* and *V. galamensis* amino acid sequences are also fatty acid epoxygenase enzymes and may be  $\Delta$ 12-epoxygenase enzymes. In this regard, the *Vernonia galamensis* amino acid sequence exemplified herein is a partial sequence which comprises only one complete histidine-rich motif (i.e. His-Arg-Asn-His-His) and a partial sequence of the first histidine-rich motif (i.e. it comprises the last two histidine residues of the His-Glu-Cys-Gly-His-His

10 motif), because the corresponding nucleotide sequence encoding same was amplified by polymerase chain reaction as a partial cDNA sequence, using a first primer to this first histidine-rich motif and a second amplification primer designed to a region upstream of the third histidine-rich motif (i.e. His-Val-Met-His-His). Additionally, the fact that the *V. galamensis* sequence was amplified using a primer specific for the first histidine-rich motif

15 indicates that the corresponding full-length sequence would also comprise this motif.

Accordingly, in a particularly preferred embodiment, the nucleic acid molecule of the invention encodes an mixed-function monooxygenase epoxygenase enzyme or like enzyme derived from *Crepis spp.*, including *Crepis palaestina* or alternatively, derived from *Vernonia*

20 *galamensis*. According to this embodiment, it is even more preferred that the subject epoxygenase at least comprises a sequence of amino acids which contains three or more histidine-rich regions as follows:

- (i) His-Glu-Cys-Gly-His-His (SEQ ID NO:15);
- (ii) His-Arg-Asn-His-His (SEQ ID NO:16); and
- 25 (iii) His-Val-Met-His-His (SEQ ID NO:17),

or a homologue, analogue or derivative thereof, wherein His designates histidine, Glu designates glutamate, Cys designates cysteine, Gly designates glycine, Arg designates arginine, Asn designates asparagine, Val designates valine, Met designates methionine.

30 The present invention clearly extends to epoxygenase genes derived from other

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species, including the epoxygenase genes derived from *Chrysanthemum spp.* and *Euphorbia lagascae*, amongst others.

In a preferred embodiment, whilst not limiting the present invention, the epoxygenase  
5 genes of other species which are encompassed by the present invention encode mixed-function monooxygenase enzymes. The present invention further extends to the isolated or recombinant polypeptides encoded by such genes and uses of said genes and polypeptides.

The invention described according to this embodiment does not encompass nucleic  
10 acid molecules which encode enzyme activities other than epoxygenase activities as defined herein, in particular the  $\Delta 12$ -desaturase enzymes derived from *Arabidopsis thaliana*, *Brassica juncea*, *Brassica napus* or *Glycine max*, amongst others, which are known to contain similar histidine-rich motifs.

15 In the present context, "homologues" of an amino acid sequence refer to those amino acid sequences or peptide sequences which are derived from polypeptides, enzymes or proteins of the present invention or alternatively, correspond substantially to the amino acid sequences listed *supra*, notwithstanding any naturally-occurring amino acid substitutions, additions or deletions thereto.

20

For example, amino acids may be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break  $\alpha$ -helical structures or  $\beta$ -sheet structures, and so on. Alternatively, or in addition, the amino acids of a homologous amino acid sequence may be  
25 replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, charge or antigenicity, and so on.

Naturally-occurring amino acid residues contemplated herein are described in Table 1.

30

A homologue of an amino acid sequence may be a synthetic peptide produced by any

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method known to those skilled in the art, such as by using Fmoc chemistry.

Alternatively, a homologue of an amino acid sequence may be derived from a natural source, such as the same or another species as the polypeptides, enzymes or proteins of the present invention. Preferred sources of homologues of the amino acid sequences listed *supra* include any of the sources contemplated herein.

"Analogues" of an amino acid sequence encompass those amino acid sequences which are substantially identical to the amino acid sequences listed *supra* notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein.

Preferred non-naturally occurring amino acids contemplated herein are listed below in Table 2.

The term "derivative" in relation to an amino acid sequence shall be taken to refer hereinafter to mutants, parts, fragments or polypeptide fusions of the amino acid sequences listed *supra*. Derivatives include modified amino acid sequences or peptides in which ligands are attached to one or more of the amino acid residues contained therein, such as carbohydrates, enzymes, proteins, polypeptides or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are also contemplated by the present invention. Additionally, derivatives may comprise fragments or parts of an amino acid sequence disclosed herein and are within the scope of the invention, as are homopolymers or heteropolymers comprising two or more copies of the subject sequences.

Procedures for derivatizing peptides are well-known in the art.

Substitutions encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as "conservative", in which case an amino acid residue is

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replaced with another naturally-occurring amino acid of similar character, for example Gly↔Ala, Val↔Ile↔Leu, Asp↔Glu, Lys↔Arg, Asn↔Gln or Phe↔Trp↔Tyr.

Substitutions encompassed by the present invention may also be "non-conservative",  
5 in which an amino acid residue which is present in a repressor polypeptide is substituted with an amino acid having different properties, such as a naturally-occurring amino acid from a different group (eg. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid.

10

Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

Amino acid deletions will usually be of the order of about 1-10 amino acid residues,  
15 while insertions may be of any length. Deletions and insertions may be made to the N-terminus, the C-terminus or be internal deletions or insertions. Generally, insertions within the amino acid sequence will be smaller than amino-or carboxyl-terminal fusions and of the order of 1-4 amino acid residues.

20 The present invention clearly extends to the subject isolated nucleic acid molecule when integrated into the genome of a cell as an addition to the endogenous cellular complement of epoxxygenase genes. Alternatively, wherein the host cell does not normally encode enzymes required for epoxy fatty acid biosynthesis, the present invention extends to the subject isolated nucleic acid molecule when integrated into the genome of said cell as an  
25 addition to the endogenous cellular genome.

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TABLE 1

Amino Acid	Three-letter Abbreviation	One-letter Symbol
5		
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
10 Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
15 Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
20 Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
25 Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

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TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
5 $\alpha$ -aminobutyric acid	Abu	L-N-methylalanine	Nmala
$\alpha$ -amino- $\alpha$ -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
10 aminoisobutyric acid	Aib	L-N-methylaspartic acid	Nmasp
aminonorbonyl- carboxylate	Norb	L-N-methylcysteine	Nmcys
cyclohexylalanine	Chexa	L-N-methylglutamine	Nmgln
cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
15 D-alanine	Dal	L-N-methylhistidine	Nmhis
D-arginine	Darg	L-N-methylisoleucine	Nmile
D-aspartic acid	Dasp	L-N-methyllucine	Nmleu
D-cysteine	Dcys	L-N-methyllysine	Nmlys
D-glutamine	Dgln	L-N-methylmethionine	Nmmet
20 D-glutamic acid	Dglu	L-N-methylnorleucine	Nmnle
D-histidine	Dhis	L-N-methylnorvaline	Nmnva
D-isoleucine	Dile	L-N-methylornithine	Nmorn
D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
D-lysine	Dlys	L-N-methylproline	Nmpro
25 D-methionine	Dmet	L-N-methylserine	Nmser
D-ornithine	Dorn	L-N-methylthreonine	Nmthr
D-phenylalanine	Dphe	L-N-methyltryptophan	Nmtrp
D-proline	Dpro	L-N-methyltyrosine	Nmtyr
D-serine	Dser	L-N-methylvaline	Nmval
30 D-threonine	Dthr	L-N-methylethylglycine	Nmetg
		L-N-methyl-t-butylglycine	Nmtbug
		L-norleucine	Nle

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D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	$\alpha$ -methyl-aminoisobutyrate	Maib
D-valine	Dval	$\alpha$ -methyl- $\gamma$ -aminobutyrate	Mgabu
D- $\alpha$ -methylalanine	Dmala	$\alpha$ -methylcyclohexylalanine	Mchexa
5 D- $\alpha$ -methylarginine	Dmarg	$\alpha$ -methylcyclopentylalanine	Mcpen
D- $\alpha$ -methylasparagine	Dmasn	$\alpha$ -methyl- $\alpha$ -naphthylalanine	Manap
D- $\alpha$ -methylaspartate	Dmasp	$\alpha$ -methylpenicillamine	Mpen
D- $\alpha$ -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D- $\alpha$ -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
10 D- $\alpha$ -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D- $\alpha$ -methylisoleucine	Dmile	N-amino- $\alpha$ -methylbutyrate	Nmaabu
D- $\alpha$ -methylleucine	Dmleu	$\alpha$ -naphthylalanine	Anap
D- $\alpha$ -methyllysine	Dmlys	N-benzylglycine	Nphe
D- $\alpha$ -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
15 D- $\alpha$ -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D- $\alpha$ -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D- $\alpha$ -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D- $\alpha$ -methylserine	Dmser	N-cyclobutylglycine	Ncbut
D- $\alpha$ -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
20 D- $\alpha$ -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D- $\alpha$ -methyltyrosine	Dmtty	N-cyclodecylglycine	Ncdec
D- $\alpha$ -methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
25 D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl) glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl) glycine	Nbhe

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D-N-methylglutamine	Dnmglu	N-(3-guanidinopropyl) glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
5 D-N-methylisoleucine	Dnmile	N-(imidazolylethyl) glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- $\gamma$ -aminobutyrate	Nmgabu
10 N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpn
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
15 N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyl-naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
$\gamma$ -aminobutyric acid	Gabu	N-( <i>p</i> -hydroxyphenyl)glycine	Nhtyr
20 L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- $\alpha$ -methylalanine	Mala
L- $\alpha$ -methylarginine	Marg	L- $\alpha$ -methylassparagine	Masn
L- $\alpha$ -methylasspartate	Masp	L- $\alpha$ -methyl- <i>t</i> -butylglycine	Mtbug
25 L- $\alpha$ -methylcysteine	Mcys	L-methylethylglycine	Metg
L- $\alpha$ -methylglutamine	Mglu	L- $\alpha$ -methylglutamate	Mglu
L- $\alpha$ -methylhistidine	Mhis	L- $\alpha$ -methylhomo phenylalanine	Mhphe
L- $\alpha$ -methylisoleucine	Mile	N-(2-methylthioethyl) glycine	Nmet
30			

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L- $\alpha$ -methylleucine	Mleu	L- $\alpha$ -methyllysine	Mlys
L- $\alpha$ -methylmethionine	Mmet	L- $\alpha$ -methylnorleucine	Mnle
L- $\alpha$ -methylnorvaline	Mnva	L- $\alpha$ -methylornithine	Morn
L- $\alpha$ -methylphenylalanine	Mphe	L- $\alpha$ -methylproline	Mpro
5 L- $\alpha$ -methylserine	Mser	L- $\alpha$ -methylthreonine	Mthr
L- $\alpha$ -methyltryptophan	Mtrp	L- $\alpha$ -methyltyrosine	Mtyr
L- $\alpha$ -methylvaline	Mval	L-N-methylhomo	
		phenylalanine	Nmhph
N-(N-(2,2-diphenylethyl)		N-(N-(3,3-diphenylpropyl)	
10 carbamylmethyl)glycine	Nnbhm	carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-			
ethylamino)cyclopropane	Nmbc		

15

A second aspect of the present invention provides an isolated nucleic acid molecule which comprises the sequence of nucleotides set forth in any one of SEQ ID NOs:1 or 3 or 5 or 19 or 20 or a complementary sequence thereto, or a homologue, analogue or derivative thereof.

20

For the purposes of nomenclature, the nucleotide sequence set forth in SEQ ID NO:1 is derived from *Crepis palaestina* and encodes the mixed function monooxygenase sequence or mixed function monooxygenase-like sequence set forth in SEQ ID NO:2. As exemplified herein, the amino acid sequence set forth in SEQ ID NO:2 has epoxygenase activity, more  
25 particularly  $\Delta$ 12-epoxygenase activity.

The nucleotide sequence set forth in SEQ ID NO: 3 corresponds to a cDNA derived from a *Crepis sp.* other than *C. palaestina* which contains high levels of vernolic acid. The amino acid sequence set forth in SEQ ID NO: 4 corresponds to the derived amino acid  
30 sequence of the *Crepis sp.* epoxygenase gene provided in SEQ ID NO:3.

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The nucleotide sequence set forth in SEQ ID NO: 5 corresponds to amplified DNA derived from *Vernonia galamensis* using amplification primers derived from a consensus sequence of mixed function monooxygenases, including the *Crepis spp.* epoxygenase gene sequences of the invention. The amplified DNA comprises a partial epoxygenase gene  
5 sequence, which includes nucleotide sequences capable of encoding the histidine-rich motif His-Arg-Asn-His-His which is characteristic of mixed function monooxygenase enzymes. The amino acid sequence set forth in SEQ ID NO: 6 corresponds to the derived amino acid sequence of the *Vernonia galamensis* epoxygenase gene provided in SEQ ID NO:5.

10 The nucleotide sequence set forth in SEQ ID NO:7 relates to the partial sequence of a *Crepis alpina* acetylenase gene which was used as a probe to isolate the nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID NO: 1. The amino acid sequence set forth in SEQ ID NO:8 corresponds to the derived amino acid sequence of said partial sequence of the *C. alpina* acetylenase gene.

15

As used herein, the term "acetylenase" shall be taken to refer to an enzyme which is capable of catalyzing the conversion of a carbon double bond in a fatty acid substrate molecule to a carbon triple bond or alternatively, which is capable of catalyzing the formation of a carbon triple bond in a fatty acid molecule.

20

The nucleotide sequence set forth in SEQ ID NO:18 corresponds to a degenerate amplification primer used to amplify putative *Euphorbia lagascae* epoxygenase gene sequences. In this regard, the nucleotide residues shown in SEQ ID NO:18 are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A  
25 represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide  
30 other than Cytosine and N represents any nucleotide residue.

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The nucleotide sequence set forth in SEQ ID NO:19 is derived from *Euphorbia lagascae* and encodes the putative cytochrome P-450-dependent monooxygenase sequence or cytochrome P-450-dependent monooxygenase-like sequence.

- 5        The nucleotide sequence set forth in SEQ ID NO: 20 is derived from *Euphorbia lagascae* and encodes a putative cytochrome P-450-dependent monooxygenase sequence or cytochrome P-450-dependent monooxygenase-like sequence.

The present invention clearly extends to the genomic gene equivalents of the cDNA  
10 molecules exemplified in any one of SEQ ID NOs: 1, 3, 5, 19 or 20.

In a most particularly preferred embodiment, the present invention provides an isolated nucleic acid molecule which comprises the nucleotide sequence set forth in any one of SEQ ID NOs: 1, 3, 5, 19 or 20 or a genomic gene equivalent of said nucleotide sequence  
15 or a homologue, analogue or derivative thereof.

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as the nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding  
20 the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

"Analogues" of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of  
25 the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

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"Derivatives" of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof.

5           Generally, homologues, analogues or derivatives of the nucleic acid molecule of the invention are produced by synthetic means or alternatively, derived from naturally-occurring sources. For example, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions as indicated *supra*.

10

          In one embodiment of the invention, preferred homologues, analogues or derivatives of the nucleotide sequences set forth in any one of SEQ ID NOs: 1, 3, 5, 19 or 20 or complementary sequences thereto, encode immunologically-active or enzymatically-active polypeptides.

15

          As used herein, the term "immunologically-active" shall be taken to refer to the ability of a polypeptide molecule to elicit an immune response in a mammal, in particular an immune response sufficient to produce an antibody molecule such as, but not limited to, an IgM or IgG molecule or whole serum containing said antibody molecule. The term "immunologically-  
20 active" also extends to the ability of a polypeptide to elicit a sufficient immune response for the production of monoclonal antibodies, synthetic Fab fragments of an antibody molecule, single-chain antibody molecule or other immunointeractive molecule.

          As used herein, the term "enzymatically-active" shall be taken to refer to the ability  
25 of a polypeptide molecule to catalyse an enzyme reaction, in particular an enzyme reaction which comprises the epoxxygenation of a carbon bond in a fatty acid substrate molecule. More particularly, whilst not limiting the invention, the term "enzymatically-active" may also refer to the ability of a polypeptide molecule to catalyse the epoxxygenation of  $\Delta$ -9 or  $\Delta$ -12 in a fatty acid substrate molecule such as linoleic acid or vernolic acid.

30

- 30 -

In an alternative embodiment, a preferred homologue, analogue or derivative of the nucleotide sequence set forth in any one of SEQ ID NOs: 1 or 3 or 5, or a complementary sequence thereto, comprises a sequence of nucleotides which is at least 65% identical to at least 20 contiguous nucleotides therein, other than a nucleotide sequence which encodes a  
5 *Crepis sp.* acetylenase enzyme.

More preferably, the percentage identity to any one of SEQ ID NOs: 1 or 3 or 5 is at least about 85%. Even more preferably, a homologue, analogue or derivative of SEQ ID NOs: 1 or 3 or 5 is at least about 90% and even more preferably at least about 95% identical  
10 to at least 100 or 250 or 500 or 1000 contiguous nucleotides therein.

The percentage identity to SEQ ID NOs: 19 or 20, or complementary sequences thereto is at least about 75% over at least about 200 contiguous nucleotides, even more preferably at least about 80%, still even more preferably at least about 90% and still even  
15 more preferably at least about 95%, including at least about 99% identity. Nucleotide sequences which are at least 65% over at least about 400 contiguous nucleotides in SEQ ID NOs: 19 or 20 are also within the scope of the invention.

Reference herein to a percentage identity or percentage similarity between two or  
20 more nucleotide or amino acid sequences shall be taken to refer to the number of identical or similar residues in a nucleotide or amino acid sequence alignment, as determined using any standard algorithm known by those skilled in the art. In particular, nucleotide and/or amino acid sequence identities and similarities may be calculated using the Gap program, which utilises the algorithm of Needleman and Wunsch (1970) to maximise the number of residue  
25 matches and minimise the number of sequence gaps. The Gap program is part of the Sequence and Analysis Software Package of the Computer Genetics Group Inc., University Research Park, Madison, Wisconsin, United States of America (Devereux *et al.*, 1984).

In a further alternative embodiment, a preferred homologue, analogue or derivative  
30 of the nucleotide sequence set forth in any one of SEQ ID NOs: 1, 3, 5, 19 or 20 or a

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complementary sequence thereto, hybridizes under at least low stringency conditions to at least 20 contiguous nucleotides derived from said sequence.

More preferably, the stringency of hybridization is at least moderate stringency, even  
5 more preferably at least high stringency.

For the purposes of defining the level of stringency, those skilled in the art will be aware that several different hybridisation conditions may be employed. For example, a low stringency may comprise a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1 %  
10 (w/v) SDS at 28°C. A moderate stringency may comprise a hybridisation and/or wash carried out in 2xSSC buffer, 0.1 % (w/v) SDS at a temperature in the range 45°C to 65°C. A high stringency may comprise a hybridisation and/or wash carried out in 0.1xSSC buffer, 0.1 % (w/v) SDS at a temperature of at least 65°C.

15 Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS in the hybridisation buffer or wash buffer and/or increasing the temperature at which the hybridisation and/or wash are performed. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification of parameters affecting hybridisation between nucleic acid molecules,  
20 reference can conveniently be made to pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

The isolated nucleic acid molecules disclosed herein may be used to isolate or identify homologues, analogues or derivatives thereof from other cells, tissues, or organ types, or  
25 from the cells, tissues, or organs of another species using any one of a number of means known to those skilled in the art.

For example, genomic DNA, or mRNA, or cDNA may be contacted, under at least low stringency hybridisation conditions or equivalent, with a hybridisation effective amount  
30 of an isolated nucleic acid molecule which comprises the nucleotide sequence set forth in any

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one SEQ ID NOs: 1, 3, 5, 19 or 20 or a complementary sequence thereto, or a functional part thereof, and hybridisation detected using a detection means.

The detection means may be a reporter molecule capable of giving an identifiable  
5 signal (e.g. a radioisotope such as  $^{32}\text{P}$  or  $^{35}\text{S}$  or a biotinylated molecule) covalently linked to the isolated nucleic acid molecule of the invention.

In an alternative method, the detection means is any known format of the polymerase chain reaction (PCR). According to this method, degenerate pools of nucleic acid "primer  
10 molecules" of about 15-50 nucleotides in length are designed based upon the nucleotide sequences disclosed in SEQ ID NOs: 1, 3, 5, 19 or 20 or a complementary sequence thereto. The homologues, analogues or derivatives (i.e. the "template molecule") are hybridized to two of said primer molecules, such that a first primer hybridizes to a region on one strand of the template molecule and a second primer hybridizes to a complementary sequence thereof,  
15 wherein the first and second primers are not hybridized within the same or overlapping regions of the template molecule and wherein each primer is positioned in a 5'- to 3'- orientation relative to the position at which the other primer is hybridized on the opposite strand. Specific nucleic acid molecule copies of the template molecule are amplified enzymatically in a polymerase chain reaction, a technique that is well known to one skilled  
20 in the art.

The primer molecules may comprise any naturally-occurring nucleotide residue (i.e. adenine, cytidine, guanine, thymidine) and/or comprise inosine or functional analogues or derivatives thereof, capable of being incorporated into a polynucleotide molecule. The nucleic  
25 acid primer molecules may also be contained in an aqueous mixture of other nucleic acid primer molecules or be in a substantially pure form.

The detected sequence may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell. Preferably, the related genetic  
30 sequence originates from another plant species.

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A third aspect of the present invention provides an isolated nucleic acid molecule which encodes the amino acid sequence set forth in any one of SEQ ID NOs: 2 or 4 or 6 or a homologue, analogue or derivative thereof.

5 In one embodiment contemplated herein, preferred homologues, analogues or derivatives of the amino acid sequences set forth in SEQ ID NOs: 2, 4, or 6 are immunologically-active or enzymatically-active polypeptides as defined *supra*.

In an alternative embodiment of the invention, preferred homologues, analogues or  
10 derivatives of the amino acid sequence set forth in any one of SEQ ID NOs: 2, 4 or 6 comprise a sequence of amino acids which is at least 60% identical thereto, other than a *Crepis sp.* acetylenase polypeptide. More preferably, homologues, analogues or derivatives of SEQ ID NOs: 2 or 4 or 6 which are encompassed by the present invention are at least about 85% identical, even more preferably at least about 90% identical and still even more  
15 preferably at least about 95% identical, and still more preferably at least about 99%-100% identical thereto.

Homologues, analogues or derivatives of any one of SEQ ID NOs: 2 or 4 or 6 may further comprise a histidine-rich region as defined *supra*. Even more preferably, the subject  
20 epoxygenase at least comprises a sequence of amino acids which contains three or more histidine rich regions as follows:

- (i) His-Glu-Cys-Gly-His-His (SEQ ID NO: 15);
- (ii) His-Arg-Asn-His-His (SEQ ID NO: 16); and
- 25 (iii) His-Val-Met-His-His (SEQ ID NO: 17),

or a homologue, analogue or derivative thereof.

The invention described according to this alternative embodiment does not encompass  
30 the  $\Delta 12$ -desaturase enzymes derived from *Arabidopsis thaliana*, *Brassica juncea*, *Brassica*

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*napus* or *Glycine max*, amongst others.

The isolated nucleic acid molecule of the present invention is useful for developing genetic constructs comprising a sense molecule wherein said genetic constructs are designed  
5 for the expression in a cell which does not normally express said nucleic acid molecule or over-expression of said nucleic acid molecule in a cell which does normally express the said nucleic acid molecule.

Accordingly, a further aspect of the invention provides a genetic construct which  
10 comprises a sense molecule which is operably connected to a promoter sequence.

The term "sense molecule" as used herein shall be taken to refer to an isolated nucleic acid molecule which encodes or is complementary to an isolated nucleic acid molecule which encodes a fatty acid epoxygenase wherein said nucleic acid molecule is provided in a format  
15 suitable for its expression to produce a recombinant polypeptide when said sense molecule is introduced into a host cell by transfection or transformation.

Those skilled in the art will be aware that a genetic construct may be used to "transfect" a cell, in which case it is introduced into said cell without integration into the  
20 cell's genome. Alternatively, a genetic construct may be used to "transform" a cell, in which case it is stably integrated into the genome of said cell.

A sense molecule which corresponds to a fatty acid epoxygenase gene sequence or homologue, analogue or derivative thereof, may be introduced into a cell using any known  
25 method for the transfection or transformation of said cell. Wherein a cell is transformed by the genetic construct of the invention, a whole organism may be regenerated from a single transformed cell, using any method known to those skilled in the art.

Thus, the epoxygenase genes described herein may be used to develop single cells or  
30 whole organisms which synthesize epoxy fatty acids not normally produced by wild or

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naturally-occurring organisms belonging to the same genera or species as the genera or species from which the transfected or transformed cell is derived, or to increase the levels of such fatty acids above the levels normally found in such wild or naturally-occurring organisms.

5

In an alternative preferred embodiment, the isolated nucleic acid molecule of the invention is capable of reducing the level of epoxy fatty acids in a cell, when expressed therein, in the antisense orientation or as a ribozyme or co-suppression molecule, under the control of a suitable promoter sequence.

10

Co-suppression is the reduction in expression of an endogenous gene that occurs when one or more copies of said gene, or one or more copies of a substantially similar gene are introduced into the cell. The present invention also extends to the use of co-suppression to inhibit the expression of an epoxygenase gene as described herein.

15

In the context of the present invention, an antisense molecule is an RNA molecule which is transcribed from the complementary strand of a nuclear gene to that which is normally transcribed to produce a "sense" mRNA molecule capable of being translated into a polypeptide. The antisense molecule is therefore complementary to the sense mRNA, or a  
20 part thereof. Although not limiting the mode of action of the antisense molecules of the present invention to any specific mechanism, the antisense RNA molecule possesses the capacity to form a double-stranded mRNA by base pairing with the sense mRNA, which may prevent translation of the sense mRNA and subsequent synthesis of a polypeptide gene product.

25

Ribozymes are synthetic RNA molecules which comprise a hybridising region complementary to two regions, each of at least 5 contiguous nucleotide bases in the target sense mRNA. In addition, ribozymes possess highly specific endoribonuclease activity, which autocatalytically cleaves the target sense mRNA. A complete description of the  
30 function of ribozymes is presented by Haseloff and Gerlach (1988) and contained in

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International Patent Application No. WO89/05852. The present invention extends to ribozymes which target a sense mRNA encoding an epoxygenase polypeptide described herein, thereby hybridising to said sense mRNA and cleaving it, such that it is no longer capable of being translated to synthesise a functional polypeptide product.

5

According to this embodiment, the present invention provides a ribozyme or antisense molecule comprising a sequence of contiguous nucleotide bases which are able to form a hydrogen-bonded complex with a sense mRNA encoding an epoxygenase described herein, to reduce translation of said mRNA. Although the preferred antisense and/or ribozyme molecules hybridise to at least about 10 to 20 nucleotides of the target molecule, the present invention extends to molecules capable of hybridising to at least about 50-100 nucleotide bases in length, or a molecule capable of hybridising to a full-length or substantially full-length epoxygenase mRNA.

15 It is understood in the art that certain modifications, including nucleotide substitutions amongst others, may be made to the antisense and/or ribozyme molecules of the present invention, without destroying the efficacy of said molecules in inhibiting the expression of the epoxygenase gene. It is therefore within the scope of the present invention to include any nucleotide sequence variants, homologues, analogues, or fragments of the said gene encoding  
20 same, the only requirement being that said nucleotide sequence variant, when transcribed, produces an antisense and/or ribozyme molecule which is capable of hybridising to the said sense mRNA molecule.

The present invention extends to genetic constructs designed to facilitate expression  
25 of a sense molecule, an antisense molecule, ribozyme molecule, or co-suppression molecule which is capable of altering the level of epoxy fatty acids in a cell.

In a particularly preferred embodiment, the sense molecule, an antisense molecule, ribozyme molecule, co-suppression molecule, or gene targeting molecule which is capable  
30 of altering the epoxy fatty acid composition of a cell derived from plant or other organism

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comprises a sequence of nucleotides set forth in any one of SEQ ID NOs: 1, 3, 5, 19 or 20 and more preferably in any one of SEQ ID NOs: 1 or 3 or 5 and even more preferably in SEQ ID NO:1 or a complementary strand, homologue, analogue or derivative thereof.

5 Those skilled in the art will also be aware that expression of a sense, antisense, ribozyme or co-suppression molecule may require the nucleic acid molecule of the invention to be placed in operable connection with a promoter sequence. The choice of promoter for the present purpose may vary depending upon the level of expression of the sense molecule required and/or the species from which the host cell is derived and/or the tissue-specificity  
10 or development-specificity of expression of the sense molecule which is required.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT  
15 box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. In the context of the present invention, the term "promoter" also includes the transcriptional regulatory sequences of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or a -10 box  
20 transcriptional regulatory sequences.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of said sense molecule in a cell. Preferred promoters may contain additional copies of one or more specific  
25 regulatory elements, to further enhance expression of the sense molecule and/or to alter the spatial expression and/or temporal expression of said sense molecule. For example, copper-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a sense molecule to confer copper inducible expression thereon.

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Placing a sense, antisense, ribozyme or co-suppression molecule under the regulatory control of a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. A promoter is usually, but not necessarily, positioned upstream or 5' of a nucleic acid molecule which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the sense, antisense, ribozyme or co-suppression molecule or chimeric gene comprising same. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in genetic constructs of the present invention include promoters derived from the genes of viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants which are capable of functioning in isolated cells or whole organisms regenerated therefrom. The promoter may regulate the expression of the sense, antisense, ribozyme or co-suppression molecule constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, pathogens, or metal ions, amongst others.

Examples of promoters include the CaMV 35S promoter, NOS promoter, octopine synthase (OCS) promoter, *Arabidopsis thaliana* SSU gene promoter, napin seed-specific promoter, P<sub>32</sub> promoter, BK5-T *imm* promoter, *lac* promoter, *tac* promoter, phage lambda  $\lambda_L$  or  $\lambda_R$  promoters, CMV promoter (U.S. Patent No. 5,168,062), T7 promoter, lacUV5

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promoter, SV40 early promoter (U.S. Patent No. 5,118,627), SV40 late promoter (U.S. Patent No. 5,118,627), adenovirus promoter, baculovirus P10 or polyhedrin promoter (U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051 and 5,169,784), and the like. In addition to the specific promoters identified herein, cellular promoters for so-called housekeeping genes are useful.

Preferred promoters according to this embodiment are those promoters which are capable of functioning in yeast, mould or plant cells. More preferably, promoters suitable for use according to this embodiment are capable of functioning in cells derived from oleaginous yeasts, oleaginous moulds or oilseed crop plants, such as flax sold under the trademark Linola® (hereinafter referred to as "Linola® flax"), sunflower, safflower, soybean, linseed, sesame, cottonseed, peanut, olive or oil palm, amongst others.

Linola® is a registered trade mark of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia.

In a more preferred embodiment, the promoter may be derived from a genomic clone encoding an epoxygenase enzyme, preferably derived from the genomic gene equivalents of epoxygenase genes derived from *Chrysanthemum spp.*, *Crepis spp.* including *C. palaestina* or other *Crepis sp.*, *Euphorbia lagascae* or *Vernonia galamensis*, which are referred to herein.

In a more preferred embodiment, the promoter may be derived from a highly-expressed seed gene, such as the napin gene, amongst others.

The genetic construct of the invention may further comprise a terminator sequence and be introduced into a suitable host cell where it is capable of being expressed to produce a recombinant polypeptide gene product or alternatively, a ribozyme or antisense molecule.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit

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which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the genetic constructs of the present invention include the nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays*, the Rubisco small subunit (SSU) gene terminator sequences, subclover stunt virus (SCSV) gene sequence terminators, any *rho*-independent *E. coli* terminator, amongst others.

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The genetic constructs of the invention may further include an origin of replication sequence which is required for replication in a specific cell type, for example a bacterial cell, when said genetic construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell.

Preferred origins of replication include, but are not limited to, the *f1*-ori and *colE1* origins of replication.

The genetic construct may further comprise a selectable marker gene or genes that are functional in a cell into which said genetic construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection

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of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp<sup>r</sup>), tetracycline resistance gene (Tc<sup>r</sup>), bacterial kanamycin resistance gene (Kan<sup>r</sup>), phosphinothricin resistance gene, neomycin phosphotransferase gene (*nptII*), hygromycin resistance gene,  $\beta$ -glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene and luciferase gene, amongst others.

10 A further aspect of the present invention provides a transfected or transformed cell, tissue, organ or whole organism which expresses a recombinant epoxygenase polypeptide or a ribozyme, antisense or co-suppression molecule as described herein, or a homologue, analogue or derivative thereof.

15 Preferably, the isolated nucleic acid molecule is contained within a genetic construct as described herein. The genetic construct of the present invention may be introduced into a cell by various techniques known to those skilled in the art. The technique used may vary depending on the known successful techniques for that particular organism.

20 Means for introducing recombinant DNA into bacterial cells, yeast cells, or plant, insect, fungal (including mould), avian or mammalian tissue or cells include, but are not limited to, transformation using CaCl<sub>2</sub> and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al.*, 1982; Paszkowski *et al.*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al.*, 1990)  
25 microparticle bombardment, electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al.*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.* (1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985).

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For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf  
5 (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5  $\mu\text{m}$  gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique,  
10 such as by precipitation.

In a particularly preferred embodiment, wherein the genetic construct comprises a "sense" molecule, it is particularly preferred that the recombinant epoxygenase polypeptide produced therefrom is enzymatically active.  
15

Alternatively, wherein the cell is derived from a multicellular organism and where relevant technology is available, a whole organism may be regenerated from the transformed cell, in accordance with procedures well known in the art.

20 Those skilled in the art will also be aware of the methods for transforming, regenerating and propagating other type of cells, such as those of fungi.

In the case of plants, plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a genetic construct of the  
25 present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue  
30 (e.g., cotyledon meristem and hypocotyl meristem).

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The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots  
5 develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

The regenerated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or  
10 T1) transformed plant may be selfed to give homozygous second generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

The regenerated transformed organisms contemplated herein may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells;  
15 clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed root stock grafted to an untransformed scion ).

A further aspect of the invention provides a method of altering the level of epoxy fatty  
20 acids in a cell, tissue, organ or organism, said method comprising expressing a sense, antisense, ribozyme or co-suppression molecule as described herein in said cell for a time and under conditions sufficient for the level of epoxy fatty acids therein to be increased or reduced.

25 In a preferred embodiment, the subject method comprises the additional first step of transforming the cell, tissue, organ or organism with the sense, antisense, ribozyme or co-suppression molecule.

As discussed *supra* the isolated nucleic acid molecule may be contained within a  
30 genetic construct.

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According to this embodiment, the cell, organ, tissue or organism in which the subject sense, antisense, ribozyme or co-suppression molecule is expressed may be derived from a bacteria, yeast, fungus (including a mould), insect, plant, bird or mammal.

5        Because a recombinant epoxygenase polypeptide may be produced in the regenerated transformant as well as *ex vivo*, one alternative preferred embodiment of the present invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising the steps of:

- 10        (i)     producing a genetic construct which comprises the cDNA or genomic epoxygenase genetic sequence of the invention placed operably under the control of a promoter capable of conferring expression on said genetic sequence in said cell, and optionally an expression enhancer element;
- (ii)     transforming said genetic construct into said cell; and
- 15        (iii)     selecting transformants which express the epoxygenase encoded by the genetic sequence at a high level.

A particularly preferred embodiment of the present invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a transgenic plant

20 comprising the steps of:

- (i)     producing a genetic construct which comprises the cDNA or genomic epoxygenase genetic sequence of the invention placed operably under the control of a seed-specific promoter and optionally an expression enhancer element, wherein said
- 25        genetic sequences is also placed upstream of a transcription terminator sequence;
- (ii)     transforming said genetic construct into a cell or tissue of said plant; and
- (iii)     selecting transformants which express the epoxygenase encoded by the genetic sequence at a high level in seeds.

30        In a more particularly preferred embodiment, the plant is an oilseed species that

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normally produces significant levels of linoleic acid, for example Linola® flax, oilseed rape, sunflower, safflower, soybean, linseed, sesame, cottonseed, peanut, olive or oil palm, amongst others.

- 5           In an even more particularly preferred embodiment, the plant is an oilseed species that normally produces significant levels of linoleic acid, for example Linola® flax, sunflower or safflower, amongst other.

Enzymatically active recombinant epoxxygenases described herein are particularly  
10 useful for the production of epoxxygenated fatty acids from unsaturated fatty acid substrates. The present invention especially contemplates the production of specific epoxxygenated fatty acids in cells or regenerated transformed organisms which do not normally produce that specific epoxxygenated fatty acid.

- 15           Accordingly, a further aspect of the invention provides a method of producing an epoxxygenated fatty acid in a cell, tissue, organ or organism, said method comprising incubating a cell, tissue, organ or organism which expresses an enzymatically active recombinant epoxxygenase of the present invention with a fatty acid substrate molecule, preferably an unsaturated fatty acid substrate molecule, for a time and under conditions  
20 sufficient for at least one carbon bond of said substrate to be converted to an epoxy group.

In an alternative embodiment, the subject method further comprises the additional first step of transforming or transfecting the cell, tissue, organ or organism with a nucleic acid molecule which encodes said recombinant epoxxygenase or a homologue, analogue or  
25 derivative thereof, as hereinbefore described. As discussed *supra* the isolated nucleic acid molecule may be contained within a genetic construct.

According to this embodiment, the cell, organ, tissue or organism in which the subject epoxxygenase is expressed is derived from a bacteria, yeast, fungus (including a mould),  
30 insect, plant, bird or mammal. More preferably, the cell, organ, tissue or organism is derived

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from a yeast, plant or fungus, even more preferably from an oleaginous yeast or plant or fungus, or from an oilseed plant which does not normally express the recombinant epoxygenase of the invention.

5        Amongst the main economic oilseed plants contemplated herein, high-linoleic genotypes of flax, sunflower, corn and safflower are preferred targets. Soybean and rapeseed are alternative targets but are less suitable for maximal epoxy fatty acid synthesis because of their lower levels of linoleic acid substrate and the presence of an active  $\Delta 15$ -desaturase competing with the epoxygenase for the linoleic acid substrate.

10

An alternative embodiment is the transformation of Linola® (= low linolenic acid flax) with the epoxygenase of the invention. Linola® flax normally contains around 70% linoleic acid with very little of this (<2%) being subsequently converted to linolenic acid by  $\Delta 15$ -desaturase (Green, 1986).

15

Preferred unsaturated fatty acid substrates contemplated herein include, but are not limited to, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, and arachidonic acid, amongst others.

20

In plant species that naturally contain high levels of vernolic acid, the  $\Delta 12$ -epoxygenase therein may be very efficient at epoxidising linoleic acid. As a consequence, the present invention particularly contemplates the expression of recombinant  $\Delta 12$ -epoxygenase derived from *Euphorbia lagascae*, *Vernonia spp.* and *Crepis spp.* at high levels in transgenic oilseeds during seed oil synthesis, to produce high levels of vernolic acid  
25 therein.

Accordingly, linoleic acid is a particularly preferred substrate according to this embodiment of the invention. Additional substrates are not excluded.

30

The products of the substrate molecules listed *supra* will be readily determined by

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those skilled in the art, without undue experimentation. Particularly preferred epoxy fatty acids produced according to the present invention include 12,13-epoxy-9-octadecenoic acid (vernolic acid) and 12,13-epoxy-9,15-octadecadienoic acid, amongst others.

5           Conditions for the incubation of cells, organs, tissues or organisms expressing the recombinant epoxygenase in the presence of the substrate molecule will vary, at least depending upon the uptake of the substrate into the cell, tissue, organ or organism, and the affinity of the epoxygenase for the substrate molecule in the particular environment selected. Optimum conditions may be readily determined by those skilled in the relevant art.

10

The present invention clearly extends to the isolated oil containing epoxy fatty acids, and/or the isolated epoxy fatty acid itself produced as described herein and to any products derived therefrom, for example coatings, resins, glues, plastics, surfactants and lubricants, amongst others.

15

The inventors have shown further that the mixed function monooxygenases (MMO) which perform catalytic functions such as desaturation, acetylenation, hydroxylation and/or epoxygenation, form a family of genes sharing considerable nucleotide and amino acid sequence similarity. For example, the desaturase, acetylenase, hydroxylase and/or  
20 epoxygenase enzymes which act on substrate molecules having a similar chain length and position of any carbon double bond(s) (if present) are more closely related to each other than to enzymes acting upon other substrates, and may be considered to be a "family".

Without being bound by any theory or mode of action, the sequence similarity between  
25 the members of any gene family has its basis in the identity of the substrate involved and the biochemical similarity of the reaction events occurring at the target carbon bond during the modification reaction, suggesting that divergent sequences within a family may comprise catalytic determinants or at least a functional part thereof which contributes to the specific catalytic properties of the family members.

30

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One example of a family is the desaturase, acetylenase, hydroxylase and/or epoxygenase enzymes which catalyse desaturation, acetylenation, hydroxylation and/or epoxygenation respectively, of the  $\Delta 12$  position of linoleic acid (hereinafter referred to as the "C18  $\Delta 12$ -MMO family"). The present inventors have compared the nucleotide and amino  
5 acid sequences of members of the C18  $\Delta 12$ -MMO family to determine the divergent regions thereof which potentially comprise the determinants of alternative catalytic functions at the  $\Delta 12$  position (hereinafter referred to as "putative catalytic determinants").

Furthermore, the presence of such families of fatty acid modifying MMOs is  
10 contemplated with respect to other fatty acid chain length and double bond positions. For example, the C18  $\Delta 15$ -desaturase is contemplated to belong to a family of related enzymes capable of desaturation, acetylenation, hydroxylation and/or epoxidation of the  $\Delta 15$  position in C18 fatty acid substrates, the C18  $\Delta 15$ -MMO family.

15 By producing synthetic genes in which these catalytic determinants have been interchanged (referred to as "domain swapping") it is possible to convert genes encoding one catalytic function into those encoding alternative catalytic functions. For example, the  $\Delta 12$  epoxygenase of the instant invention may be converted to a  $\Delta 12$  acetylenase by replacing portions of its C-terminal and N-terminal sequences with the equivalent domains from the  
20 *Crepis alpina*  $\Delta 12$  acetylenase. Similarly, the reverse domain swapping may also be performed.

As a further refinement, such changes in catalytic function can similarly be effected by making specific changes (e.g. addition, substitution or deletion) to only those amino-acids  
25 within each domain that are critical for determining the relevant catalytic function (such as by site-directed mutagenesis).

Accordingly, a further aspect of the present invention contemplates a synthetic fatty acid gene comprising a sequence of nucleotides derived from an epoxygenase gene as  
30 described herein, wherein said synthetic fatty acid gene encodes a polypeptide with

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epoxygenase or acetylenase or hydroxylase or desaturase activity, wherein said polypeptide either comprises an amino acid sequence which differs from a naturally-occurring epoxygenase or acetylenase or hydroxylase or desaturase enzyme, or said polypeptide exhibits catalytic properties which are different from a naturally-occurring epoxygenase or acetylenase  
5 or hydroxylase or desaturase enzyme or said polypeptide comprises a sequence of amino acids which are at least about 60% identical to a part of SEQ ID NO: 2 or 4 or 6 or homologue, analogue or derivative of said part.

Preferably, the synthetic fatty acid gene of the invention is derived from a  $\Delta 12$   
10 epoxygenase gene.

In one embodiment, the synthetic fatty acid gene of the invention encodes a fusion polypeptide in which the N-terminal and/or C-terminal amino acids of any one of SEQ ID NOs: 2 or 4 or 6 are replaced, in-frame, by amino acid sequences of a different member of  
15 the same family.

In a particularly preferred embodiment, the N-terminal and/or C-terminal amino acids of SEQ ID NO: 2 or 4 or 6 are replaced by the corresponding regions of the acetylenase, desaturase or hydroxylase polypeptides set forth in Figure 2. More preferably, at least about  
20 30 amino acid residues from the N-terminal and/or C-terminal regions of any one of SEQ ID NOs: 2 or 4 or 6 are replaced, in-frame, by the corresponding regions of the acetylenase, desaturase or hydroxylase polypeptides set forth in Figure 2.

In an alternative embodiment, the synthetic fatty acid gene of the invention encodes  
25 a fusion polypeptide in which the N-terminal and/or C-terminal amino acids of a fatty acid acetylenase or fatty acid hydroxylase or fatty acid desaturase are replaced, in-frame, by the N-terminal and/or C-terminal region of any one of SEQ ID NOs: 2 or 4 or 6.

In a particularly preferred embodiment, the N-terminal and/or C-terminal amino acids  
30 of a fatty acid acetylenase or fatty acid hydroxylase or fatty acid desaturase are replaced, in-

- 50 -

frame, by the N-terminal and/or C-terminal region of any one of SEQ ID NOs: 2 or 4 or 6. Even more preferably, the fatty acid acetylenase or fatty acid hydroxylase or fatty acid desaturase is selected from the list set forth in Figure 2.

5 Even still more preferably, at least about 30 amino acid residues from the N-terminal and/or C-terminal regions of a fatty acid acetylenase or fatty acid hydroxylase or fatty acid desaturase are replaced, in-frame, by the N-terminal and/or C-terminal region of any one of SEQ ID NOs: 2 or 4 or 6.

10 Accordingly, the present invention extends to any variants of the epoxygenase enzymes referred to herein, wherein said variants are derived from an epoxygenase polypeptide as described herein and exhibit demonstrable acetylenase or hydroxylase or desaturase activity, and either comprises an amino acid sequence which differs from a naturally-occurring acetylenase or hydroxylase or desaturase enzyme, or exhibit catalytic  
15 properties which are different from a naturally-occurring acetylenase or hydroxylase or desaturase enzyme, or comprise a sequence of amino acids which are at least about 60% identical to any one of SEQ ID NOs: 2 or 4 or 6.

As with other aspects of the invention, the variants described herein may be produced  
20 as recombinant polypeptides or in transgenic organisms, once the subject synthetic genes are introduced into a suitable host cell and expressed therein.

The recombinant polypeptides described herein or a homologue, analogue or derivative thereof, may also be immunologically active molecules.

25

A further aspect of the present invention provides an immunologically-interactive molecule which is capable of binding to a recombinant epoxygenase polypeptide of the invention.

30 Preferably, the recombinant epoxygenase polypeptide to which the immunologically-

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interactive molecule is capable of binding comprises a sequence of amino acids set forth in any one of SEQ ID NOs: 2, 4 or 6, or a homologue, analogue or derivative thereof.

In one embodiment, the immunologically interactive molecule is an antibody molecule.

5 The antibody molecule may be monoclonal or polyclonal. Monoclonal or polyclonal antibodies may be selected from naturally occurring antibodies to an epitope, or peptide fragment, or synthetic epoxygenase peptide derived from a recombinant gene product or may be specifically raised against a recombinant epoxygenase or a homologue, analogue or derivative thereof.

10

Both polyclonal and monoclonal antibodies are obtainable by immunisation with an appropriate gene product, or epitope, or peptide fragment of a gene product. Alternatively, fragments of antibodies may be used, such as Fab fragments. The present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is  
15 considered herein to include fragments and hybrids of antibodies

The antibodies contemplated herein may be used for identifying genetic sequences which express related epoxygenase polypeptides encompassed by the embodiments described herein.

20

The only requirement for successful detection of a related epoxygenase genetic sequence is that said genetic sequence is expressed to produce at least one epitope recognised by the antibody molecule. Preferably, for the purpose of obtaining expression to facilitate detection, the related genetic sequence is placed operably behind a promoter sequence, for  
25 example the bacterial *lac* promoter. According to this preferred embodiment, the antibodies are employed to detect the presence of a plasmid or bacteriophage which expresses the related epoxygenase. Accordingly, the antibody molecules are also useful in purifying the plasmid or bacteriophage which expresses the related epoxygenase.

30 The subject antibody molecules may also be employed to purify the recombinant

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epoxygenase of the invention or a naturally-occurring equivalent or a homologue, analogue or derivative of same.

The present invention is further described by reference to the following non-limiting  
5 Examples.

### EXAMPLE 1

#### Characterization of epoxy fatty acids in *Euphorbia lagascae* and *Crepis spp.*

10 Seed from the wild species *Euphorbia lagascae* and from various *Crepis* species were screened by gas liquid chromatography for the presence of epoxy fatty acids.

As shown in Table 3, *Euphorbia lagascae* contains very high levels of the epoxy fatty acid vernolic acid in its seed oil. Seeds from *Crepis palaestina* were shown to contain 61.4  
15 weight % of vernolic acid and 0.71 weight % of the acetylenic fatty acid crepyninic acid of total fatty acids (Table 3).

TABLE 3

Fatty acid composition of lipids derived from seeds of  
20 *Crepis alpina*, *Crepis palaestina* and *Euphorbia lagascae*

Fatty acid	Relative distribution (weight %) <sup>a</sup>		
	<i>Crepis alpina</i>	<i>Crepis palaestina</i>	<i>Euphorbia lagascae</i>
25 Palmitic	3.9	5.1	4.3
Stearic	1.3	2.3	1.8
Oleic	1.8	6.3	22.0
Linoleic	14.0	23.0	10.0
Crepynic	75.0	0.7	0
30 Vernolic	0	61.4	58.0
Other	4.0	1.2	3.9

<sup>a</sup> Calculated from the area % of total integrated peak areas in gas liquid chromatographic determination of methyl ester derivatives of the seed lipids

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**EXAMPLE 2****Biochemical characterization of linoleate  $\Delta$ 12-epoxygenases in  
*Euphorbia lagascae* and *Crepis palaestina***

5       The enzyme, linoleate  $\Delta$ 12-epoxygenase synthesizes vernolic acid from linoleic acid. Linoleate  $\Delta$ 12-epoxygenases derived from *Euphorbia lagascae* and *Crepis palaestina* are localized in the microsomes. The enzymes from these species at least can remain active in membrane (microsomal) fractions prepared from developing seeds.

10       Preparations of membranes from *Euphorbia lagascae* and assays of their epoxygenase activities were performed as described by Bafor *et al.* (1993) with incubations containing NADPH, unless otherwise indicated in Table 4. Lipid extraction, separation and methylation as well as GLC and radio-GLC separations were performed essentially as described by Kohn *et al.* (1994) and Bafor *et al.* (1993).

15       Preparations of membranes from *Crepis alpina* and *Crepis palaestina* were obtained as follows. *Crepis alpina* and *Crepis palaestina* plants were grown in green houses and seeds were harvested at the mid-stage of development (17-20 days after flowering). Cotyledons were squeezed out from their seed coats and homogenised with mortar and pestle  
20 in 0.1M phosphate buffer, pH 7.2 containing 0.33M sucrose, 4 mM NADH, 2 mM CoASH, 1 mg of bovine serum albumin/ml and 4,000 units of catalase/ml. The homogenate was centrifuged for 10 min at 18,000 x g and the resulting supernatant centrifuged for 60 min at 150,000 x g to obtain a microsomal pellet.

25       Standard desaturase, acetylenase and epoxygenase assays with microsomal membranes from *Crepis* species were performed at 25°C with microsomal preparations equivalent to 0.2mg microsomal protein resuspended in fresh homogenisation buffer and 10 nmol of either [1-<sup>14</sup>C]18:1-CoA or [1-<sup>14</sup>C]18:2-CoA (specific activity 85,000 d.p.m./nmol) in a total volume of 360 $\mu$ l. When NADPH was used as coreductant, the membranes were resuspended in  
30 homogenisation buffer where NADH had been replaced by NADPH.

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Biochemical characterisation of the microsomal linoleate  $\Delta$ 12-epoxygenase derived from *Euphorbia lagascae* and *Crepis palaestina* was carried out and data obtained were compared to the biochemical characteristics of oleate  $\Delta$ 12-desaturase and linoleate  $\Delta$ 12-  
5 acetylenase enzymes derived from microsomal preparations of *Crepis alpina* (Table 4).

As shown in Table 4, the *Crepis palaestina* linoleate  $\Delta$ 12-epoxygenase exhibits similar biochemical features to the linoleate  $\Delta$ 12-acetylenase and oleate  $\Delta$ 12-desaturase from *Crepis alpina*, in so far as all three enzymes require  $O_2$ , work equally well with either NADH or  
10 NADPH as the coreductants, and are inhibited by cyanide but not by carbon monoxide. Additionally, none of these enzymes are inhibited by monoclonal antibodies against cytochrome P450 reductase.

The data in Table 4 suggest that the *Crepis palaestina* linoleate  $\Delta$ 12-epoxygenase  
15 belongs to the same class of enzyme as the *Crepis alpina* microsomal oleate  $\Delta$ 12-desaturase and linoleate  $\Delta$ 12-acetylenase.

In contrast, the *Euphorbia lagascae* linoleate  $\Delta$ 12-epoxygenase requires NADPH as the coreductant, is not inhibited by cyanide, but is inhibited by carbon monoxide (Table 4).  
20 Additionally, the inventors have discovered that the *Euphorbia lagascae* linoleate  $\Delta$ 12-epoxygenase is inhibited by monoclonal antibodies raised against a cytochrome P450 reductase enzyme. These data suggest that the *Euphorbia lagascae* linoleate  $\Delta$ 12-epoxygenase belongs to the cytochrome P450 class of proteins and is therefore not related biochemically to the *Crepis palaestina* linoleate  $\Delta$ 12-epoxygenase.

25

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TABLE 4

Comparison of the biochemical characteristics of epoxygenases, acetylenases and desaturases derived from *Crepis spp.* and *Euphorbia lagascae*

Treatment	Enzyme Activity (% of control)			
	<i>C. alpina</i> oleate $\Delta$ 12- desaturase	<i>C. alpina</i> linoleate $\Delta$ 12- acetylenase	<i>C. palaestina</i> linoleate $\Delta$ 12- epoxygenase	<i>E. lagascae</i> linoleate $\Delta$ 12- epoxygenase
Carbon monoxide	85	84	88	3
Anti-P450 reductase antibodies ( $C_5A_5$ )	96	91	94	33
KCN	16	0	35	92
minus NADH plus NADPH	95	73	94	100 (control)
minus NADPH plus NADH	100 (control)	100 (control)	100 (control)	11

## EXAMPLE 3

Strategy for cloning *Crepis palaestina* epoxygenase genes

Cloning of the *Crepis palaestina* epoxygenase genes relied on the characteristics of the *C. palaestina* and *C. alpina* enzymes described in the preceding Examples.

In particular, poly (A)+ RNA was isolated from developing seeds of *Crepis palaestina* using a QuickPrep Micro mRNA purification kit (Pharmacia Biotechnology) and used to synthesise an oligosaccharide d(T)-primed double stranded cDNA. The double

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stranded cDNA was ligated to *EcoRI/NotI* adaptors (Pharmacia Biotechnology) and a cDNA library was constructed using the ZAP-cDNA Gigapack cloning kit (Stratagene).

Single-stranded cDNA was prepared from RNA derived from the developing seeds  
5 of *Crepis alpina*, using standard procedures. A PCR fragment, designated as D12V (SEQ ID NO:7), was obtained by amplifying the single-stranded cDNA using primers derived from the deduced amino acid sequences of plant mixed-function monooxygenases.

The D12V fragment was subsequently random-labelled and used to screen the *Crepis*  
10 *palaestina* cDNA library *supra* on Hybond N<sup>+</sup> membrane filters from Amersham as prescribed by the manufacturer using standard hybridization conditions. This approach resulted in the purification of a recombinant bacteriophage, designated Cpa12.

The nucleotide sequence of the Cpa12 cDNA was determined and is set forth in SEQ  
15 ID NO: 1.

The Cpa12 cDNA appeared to be full-length. A schematic representation of an expression vector comprising the Cpa12 cDNA is presented in Figure 1. The genetic construct set forth therein is designed for introduction into plant material for the production  
20 of a transgenic plant which expresses the subject epoxygenase. Those skilled in the art will recognise that similar expression vectors may be produced, without undue experimentation, and used for the production of transgenic plants which express any of the genetic sequences of the instant invention, by replacing the Cpa12 cDNA with another structural gene sequence.

25 As shown in Figure 2, the nucleotide sequence of the Crep1 cDNA encoded a polypeptide which was closely related at the amino acid level, at least, to an acetylenase enzyme of *C. alpina* (Bafor *et al.* 1997; International Patent Application No. PCT/SE97/00247).

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The 1.4 kb insert from pCpa12 was sequenced (SEQ ID NO. 1) and shown to comprise an open reading frame which encodes a polypeptide of 374 amino acids in length. The deduced amino acid sequence of Cpa12 showed 81% identity and 92% similarity to the  $\Delta 12$ -acetylenase from *Crepis alpina* and approximately 60% identity and 80% similarity with plant microsomal  $\Delta 12$ -desaturase proteins (Figure 2). However, the polypeptide encoded by Cpa12 comprised significant differences in amino acid sequence compared to non-epoxygenase enzymes. In particular, the Cpa12 has a deletion of six contiguous amino acids in the 5' terminal region compared to all the microsomal  $\Delta 12$  desaturases, and a deletion of two contiguous amino acids in the 3' terminal region compared to the Crep1  $\Delta 12$  acetylenase (Figure 2).

Although membrane-bound fatty acid desaturase genes show limited sequence homologies, they all contain three regions of conserved histidine-rich motifs as follows:

- (i) His-(Xaa)<sub>3-4</sub>-His;
- (ii) His-(Xaa)<sub>2-3</sub>-His-His; and
- (iii) His-(Xaa)<sub>2-3</sub>-His-His,

wherein His designates histidine, Xaa designates any naturally-occurring amino acid residue as set forth in Table 1 herein, the integer (Xaa)<sub>3-4</sub> refers to a sequence of amino acids comprising three or four repeats of Xaa, and the integer (Xaa)<sub>2-3</sub> refers to a sequence of amino acids comprising two or three repeats of Xaa. These histidine-rich regions are suggested to be a part of the active centre of the enzyme (Shanklin *et al.*, 1994).

The amino acid sequence encoded by the Cpa12 cDNA comprises three histidine-rich motifs similar, but not identical, to the histidine-rich motifs of the  $\Delta 12$ -desaturase enzymes. These data suggest that the Cpa12 cDNA encodes an enzyme which belongs to the mixed function monooxygenase class of enzymes.

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The analysis of fatty acids presented in Example 1 *supra* indicated that vernolic acid was at least present in the seeds of *Crepis palaestina*. This enzyme may in fact be present exclusively in the seeds of *C. palaestina*. The expression of the Cpal2 gene was examined using the 3' untranslated region of the Cpal2 cDNA clone as a hybridisation probe on  
5 northern blots of mRNA derived from developing seeds and leaves of *C. palaestina*. As shown in Figure 3, the Cpal2 gene was highly-expressed in developing seeds but no expression could be detected in leaves. These data are consistent with the enzyme activity profile of *C. palaestina* linoleate  $\Delta$ 12-epoxygenase in these tissues.

10

#### EXAMPLE 4

##### Strategy for cloning *Euphorbia lagascae* epoxygenase genes

Cloning of the *Euphorbia lagascae* epoxygenase genes relied on the characteristics of the *E. lagascae* enzymes as described in the preceding Examples.

15

In one approach taken to clone *Euphorbia lagascae* epoxygenase genes, RNA was collected from immature embryos of *Euphorbia lagascae* taken at a stage of active vernolic acid synthesis and used to construct a cDNA library. The cDNA library was constructed in the Lambda Zap II vector (Stratagene) as described in the preceding Example, with the  
20 exception that the cDNA inserts were cloned in a directional manner into *Eco*RI-*Xho*I sites of the plasmid vector embedded in the lambda vector.

The degenerate PCR primer set forth in Figure 4 (SEQ ID NO:18) was synthesised and used to amplify nucleotide sequences which encode P450 enzyme sequences from the  
25 *Euphorbia lagascae* cDNA library. For PCR amplification reactions, an aliquot 100  $\mu$ l of the cDNA library was extracted with phenol:chloroform [1:1(v/v)] and DNA was precipitated by the addition of 2 volumes of ethanol and finally resuspended in 100  $\mu$ l of water. An aliquot (1  $\mu$ l) of the resuspended DNA was used as template in a PCR amplification reaction. PCR reactions were performed in 10  $\mu$ l of *Taq*I polymerase buffer containing  
30 200  $\mu$ M of each dNTP, 10 pmol of the degenerate primer, 1 pmol of T7 polymerase promoter

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primer and 0.4 units of *TaqI* polymerase.

The amplification conditions were 2 min at 94°C, and five cycles, each cycle comprising 1 min at 48°C followed by 2 min at 72°C followed by 30 sec at 93°C, then 28  
5 cycles, each cycle comprising 30 sec at 55°C followed by 90 sec at 72°C followed by 30 sec at 93°C, and finally one cycle comprising 30 sec at 55°C followed by 10 min at 72°C followed by 1 min at 25°C.

PCR products were purified and digested using *EcoRI* and *XhoI*, and then sub-cloned  
10 into Bluescript vector for sequence characterisation. One of the PCR clones was found to encode a P450 sequence and was used as a probe to isolate a full-length cDNA clone. This nucleotide sequence is set forth in SEQ ID NO:19. SEQ ID NO:19 had similarity to other members of the 2C family of P450 genes. In particular, SEQ ID NO:19 shows on average a 40% identity to the human and rat arachidonic epoxygenase sequences using the BLAST  
15 program.

Additionally, the SEQ ID NO:19 transcript was shown to be expressed in seeds of *Euphorbia lagascae* but not in roots or leaves (Figure 5B). The SEQ ID NO:19 transcript was detected in the developing seeds of *Vernonia galamensis* but not in those of *E. cyparissis*  
20 or flax, two species that do not produce epoxy fatty acids (Figures 5A and 5B).

In an alternative approach taken to clone *Euphorbia lagascae* epoxygenase genes, subtractive hybridization strategy was employed to isolate genes that are specifically expressed in an organism which produces high levels of epoxy fatty acids.

25

In particular, the subtractive hybridization method described in Figure 6 was employed to isolate epoxygenase genes which are expressed specifically in *Euphorbia lagascae*, which produces high levels of the epoxy fatty acid, vernolic acid (Example 1) and not in the closely related species *Euphorbia cyparissus*, which does not produce vernolic acid.

30

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Accordingly, mRNA was isolated from developing embryos of *Euphorbia lagascae* at a stage where they are actively synthesising vernolic acid and used to generate so-called "tester" cDNA. Additionally, mRNA was isolated from the developing embryos of *E. cyparissis* (at a similar stage of development to *E. lagascae*) and used to generate so-called

5 "driver" cDNA.

The subtractive hybridization procedure led to a library which was enriched for sequences exclusively expressed in *Euphorbia lagascae*. Clones from this library were sequenced and at least two sequences were identified as encoding P450 proteins based on

10 similarity to other P450 sequences in the database. These two P450 PCR clones were used as probes to isolate the corresponding full length cDNA clones from the cDNA library referred to earlier.

One of the isolated P450 cDNAs, comprising the sequence of nucleotides set forth in

15 SEQ ID NO:20, appeared to be expressed in tissues of *Euphorbia lagascae* (Figure 7B) and no homologous transcripts were detected in seed tissue of *E. cyparissis* or flax, two species that do not produce epoxy fatty acids. The deduced amino acid sequence of SEQ ID NO:20 indicates that the cDNA clone is full-length and encodes a P450 enzyme. These data suggest that the cDNA exemplified by SEQ ID NO:20 may encode an epoxygenase, for example the

20 linoleate  $\Delta$ 12-epoxygenase which converts linoleic acid to vernolic acid.

### EXAMPLE 5

#### Demonstration of epoxygenase activity

25 Confirmation that the cDNA clones exemplifying the invention encode epoxygenase activities was obtained by transforming *Arabidopsis thaliana*, which does not produce epoxy fatty acids, in particular vernolic acid, with each individual candidate clone and examining transformed tissue for the presence of epoxygenated fatty acids which they would not otherwise produce, or for hydroxy fatty acids which might be formed from the metabolism

30 of an epoxygenated fatty acid by the action of endogenous epoxide hydrolases (Blee and

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Schuber, 1990).

The epoxygenase cDNA comprising SEQ ID NO:1 was cloned into the Binary vector construct set forth in Figure 8. Briefly, the cDNA sequence was sub-cloned from the pCpal2  
5 plasmid (Figure 1) into the binary plasmid, by digesting pCpal2 with *EcoRI* and end-filling the restriction fragment using T4 DNA polymerase enzyme. The Binary vector (Figure 8) was linearised using *BamHI* and also end-filled using T4 DNA polymerase. For the end-filling reactions, 1µg of cDNA insert or linearised Binary vector DNA was resuspended in 50µl of  
10 T4 DNA polymerase buffer (33mM Tris-acetate pH 7.9, 66mM potassium acetate, 10mM magnesium acetate and 5mM DDT) supplemented with 100mM of each dNTP and 0.1mg/ml BSA and 3 units of T4 DNA polymerase, and incubated for 6 min incubation at 37°C. The reaction was stopped by heating at 75°C for 10mins. The blunt-ended cDNA and Binary vector DNA were ligated using T4 DNA ligase and standard ligation conditions as recommended by Promega. Clones were selected in which the SEQ ID NO: 1 sequence was  
15 inserted behind the napin promoter, in the sense orientation, thereby allowing for expression of the epoxygenase polypeptide. The Binary plasmid harbouring SEQ ID NO: 1, in the sense orientation, operably under control of the truncated napin promoter, is represented schematically in Figure 9.

20 The Binary plasmid set forth in Figure 9 was transformed into *Agrobacterium* strain AGL1 using electroporation and used to transform *Arabidopsis thaliana*. Transgenic *A. thaliana* plants were obtained according to the method described by Valvekens *et al.* (1988) and Dolferus *et al.* (1994).

25 Transgenic plants and untransformed (i.e. control) plants were grown to maturity. Mature seed of each plant was analysed for fatty acid composition by standard techniques. Primary transformant (T<sub>0</sub>) plants were established and T1 seed was harvested from each plant and analysed for fatty acid composition by gas chromatography. Twelve T<sub>0</sub> plants were shown to contain vernolic acid in their T1 seed lipids at concentrations ranging from 0.9%  
30 to 15.8% of total fatty acids, while untransformed control plants contained no vernolic acid

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(Table 5). The highest-expressing plant line was Cpal-17, for which the GLC elution profiles (from packed column and capillary column analysis) is presented in Figure 10. The GLC elution profile from packed column for the untransformed control is also shown in Figure 10.

5

**TABLE 5**  
**Vernolic acid levels in transgenic *A. thaliana***  
**lines expressing SEQ ID NO:1**

10

15

20

<b>T<sub>0</sub> Plant No.</b>	<b>Vernolic acid (weight % of total seed fatty acids)</b>
Cpal-4	1.4
Cpal-5	1.1
Cpal-8	2.7
Cpal-9	0.9
Cpal-13	0.9
Cpal-15	1.1
Cpal-17	15.8
Cpal-21	1.3
Cpal-23	1.4
Cpal-24	1.0
Cpal-25	1.2
Cpal-26	1.1
<b>untransformed control line</b>	<b>0.0</b>

25 Alternatively, or in addition, putative fatty acid epoxigenase sequences described herein are each transformed into *Linum usitatissimum* (flax) and *Arabidopsis thaliana* under the control of the napin seed-specific promoter. Transgenic flax and *Arabidopsis thaliana* plants are examined for presence of epoxy fatty acids in developing seed oils. Previous work

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has shown that if epoxy fatty acids are fed to developing flax embryos they are incorporated into triglycerides (Example 10).

Alternatively, yeast are also transformed with the epoxxygenase clones of the invention  
5 and assayed for production of epoxy fatty acids.

### EXAMPLE 6

#### Mass spectroscopy confirmation of epoxy fatty acids in T<sub>1</sub> *Arabidopsis* seed borne on primary T<sub>0</sub> transgenic plants

10

Gas chromatography of methyl esters prepared from seed lipids of T<sub>1</sub> seed of *Cpal2*-transformed *Arabidopsis thaliana* plants (Example 5) revealed the presence of two additional fatty acids compared to the untransformed controls. The first of these compounds had a retention time equivalent to that of a vernolic acid standard. The second compound had a  
15 longer retention time and was putatively identified as 12,13-epoxy-9,15-octadecadienoic acid, an expected derivative of vernolic acid, resulting from desaturation at the  $\Delta 15$  position by the endogenous *Arabidopsis thaliana*  $\Delta 15$ -desaturase.

Confirmation of the exact identity of the two peaks was obtained by mass spectroscopy  
20 of diols which were prepared from the epoxy fatty acid fraction derived from *Cpal2*-transformed plants. The diols were converted further to trimethylsilyl ethers and analysed by GC-MS DB23 on a fused silica capillary column (Hewlett-Packard 5890 II GC coupled to a Hewlett Packard 5989A MS working in electron impact at 70eV15). The total ion chromatogram showed two peaks as follows:

- 25 (i) The first eluting peak had prominent ions of mass 73, 172, 275, and 299, indicating that the epoxy group was positioned at C-12 of a C18 fatty acid and that a double bond occurred between the epoxy group and the carboxyl terminus. This mass spectra was identical to the spectra of a trimethylsilyl ether derivative of diols prepared from pure vernolic acid (12,13-epoxy-9-octadecenoic acid); and

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(ii) The second eluting peak had prominent ions of mass 73, 171, 273, and 299, indicating the presence of two double bonds and an epoxy group positioned at C-12 of a C18 fatty acid, consistent with the mass spectrum for 12,13-epoxy-9,15-octadecadienoic acid.

5

### EXAMPLE 7

#### Fatty acid analysis of *Cpal2* transgenic *Arabidopsis* plants

The T1 seed derived from transformed *Arabidopsis thaliana* plants expressing the *Cpal2* cDNA clone under control of the napin promoter was germinated and T1 plants were  
10 established from five T<sub>0</sub> lines (Nos. 4, 8, 13, 17 & 21 in Table 5). The T2 seed was harvested from each T1 plant and analysed for fatty acid composition. The progeny of transformant Nos. 4, 8, 13 and 21 (Table 5) segregated as expected for presence of vernolic acid, with those plants containing vernolic acid ranging up to 3.1% (Table 6).

All T1 plants that contained vernolic acid (i.e. epoxy 18:1 in Table 6) also contained  
15 12,13-epoxy-9,15-octadecadienoic acid (i.e. epoxy 18:2 in Table 6; see also Figure 11), indicating that some of the vernolic acid synthesised by the *Cpal2* epoxygenase was subsequently desaturated by the endogenous  $\Delta 15$ -desaturase.

TABLE 6

Fatty acid composition of selfed seeds borne on T<sub>1</sub> plants derived from five primary *Cpal2* transformants of *Arabidopsis thaliana*

Plant  No.	Fatty Acid										
	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
4-1	8.3	3.9	15.5	23.9	20.6	2.8	16.5	1.7	1.6	-	-
4-2	7.6	4.1	20.3	17.8	18.0	3.4	19.7	1.8	2.0	0.82	0.63
4-3	8.4	4.3	26.0	13.5	16.1	2.8	19.0	1.8	1.6	2.03	0.72
4-4	7.6	4.0	25.2	14.3	16.0	2.8	19.8	2.1	1.7	1.99	0.92
4-5	7.2	3.6	15.6	23.1	19.9	3.1	19.7	1.6	2.1	-	-
4-6	7.0	3.7	19.2	17.8	18.4	3.2	20.3	1.9	2.1	0.87	0.33
4-8	7.4	3.9	16.0	23.6	20.1	3.1	18.7	1.6	1.8	-	-
4-9	7.6	4.0	24.8	13.4	15.9	2.8	20.4	2.3	1.8	2.30	1.07
4-10	7.6	4.2	24.0	13.5	16.2	3.1	20.4	1.9	1.8	1.97	0.83
4-11	7.4	3.9	15.0	23.2	20.4	3.3	18.8	1.7	2.0	-	-
4-12	8.7	4.0	20.7	17.0	17.5	2.6	17.2	1.7	1.5	1.38	0.74
4-13	7.2	4.1	21.9	16.4	17.7	3.2	21.0	1.7	1.9	1.14	0.45
8-1	8.1	3.9	26.1	15.0	16.0	2.6	19.5	2.0	1.6	1.79	0.82
8-3	8.7	4.2	31.6	11.5	14.0	2.2	18.5	1.9	1.4	2.38	1.13
8-4	8.5	4.1	27.2	15.1	16.1	2.5	18.9	1.8	1.4	1.70	0.84
8-5	9.1	4.2	27.7	14.7	16.2	2.4	18.3	1.7	1.5	1.70	0.82
8-6	9.8	4.0	26.0	17.2	17.2	2.3	16.9	1.6	1.2	1.36	0.71
8-7	10.0	3.5	15.2	25.3	22.3	2.3	14.4	1.7	1.7	-	-
8-8	8.4	4.3	32.2	10.7	13.3	2.5	20.3	1.6	1.5	1.92	0.82
8-9	9.8	3.6	15.9	25.3	22.0	2.4	14.5	1.6	1.3	-	-
8-10	7.5	3.9	24.4	15.9	15.8	2.8	20.2	2.2	1.8	1.70	0.82
8-11	7.6	3.8	15.4	23.6	19.8	2.9	19.4	1.5	1.8	-	-
8-12	9.4	3.7	24.2	16.7	16.7	2.2	17.6	0.9	1.2	1.46	0.65
8-13	10.3	4.3	25.3	17.1	17.9	2.2	16.0	1.8	1.3	1.48	0.73
13-1	7.0	4.3	33.3	8.1	11.1	2.7	23.1	1.7	1.6	2.42	1.26
13-2	7.2	4.3	30.4	9.6	12.7	2.8	22.0	1.8	1.6	2.48	1.37
13-3	7.6	3.9	15.6	23.6	19.7	3.0	19.1	1.7	1.8	-	-
13-4	7.7	4.0	15.2	22.5	19.3	3.1	18.0	1.6	1.7	-	-
13-5	8.0	4.2	16.3	22.2	17.5	4.4	19.4	2.0	2.0	-	-

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Plant No.	Fatty Acid										
	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
13-6	7.9	4.4	25.7	14.7	15.8	2.9	21.2	1.6	1.7	1.56	0.63
13-7	7.9	4.0	16.0	23.3	19.6	3.0	19.1	1.6	1.8	-	-
13-9	8.0	4.0	16.1	23.6	20.0	2.9	18.7	1.6	1.6	-	-
13-10	8.7	4.2	34.6	9.6	12.5	2.2	19.1	1.5	1.2	2.21	1.01
13-11	8.7	4.0	17.6	24.3	18.9	2.8	17.1	1.6	1.4	-	-
13-12	8.9	4.2	26.4	14.6	16.0	2.5	17.5	1.6	1.2	1.62	0.74
13-13	9.0	4.4	27.9	14.4	15.3	2.5	18.9	1.5	1.4	1.30	0.77
13-14	9.2	4.2	17.2	23.8	18.8	2.7	17.9	1.7	1.5	-	-
13-15	8.4	4.2	19.7	20.9	18.6	2.7	17.7	1.4	1.5	0.40	0.16
13-16	8.2	4.3	23.0	17.1	17.3	2.8	19.3	1.5	1.5	0.97	0.42
13-17	8.3	4.1	15.7	23.9	19.9	2.8	17.6	1.6	1.9	-	-
17-1	7.6	4.1	15.8	23.7	19.6	2.6	20.3	1.7	1.7	-	-
17-2	8.3	4.1	16.4	24.4	20.1	2.3	16.8	1.5	1.4	-	-
17-3	8.1	4.1	16.4	24.3	20.0	2.5	17.6	1.6	1.4	-	-
21-1	8.1	4.3	26.9	14.5	15.0	2.9	19.9	1.5	1.5	1.64	0.63
21-2	8.2	4.0	27.9	11.8	13.2	2.5	19.8	1.7	1.5	2.18	0.91
21-3	8.8	3.7	16.4	24.4	20.6	2.5	17.3	1.7	1.4	-	-
21-4	7.9	3.9	19.6	19.8	17.8	2.7	18.7	1.7	1.7	0.66	0.46
21-5	7.2	4.2	26.5	12.9	14.4	3.0	21.5	0.9	1.8	1.78	0.84
21-6	8.3	4.2	27.4	13.9	15.4	2.6	19.9	1.7	1.5	1.66	0.65
21-7	7.2	4.2	26.8	13.5	13.4	3.0	21.9	1.7	1.8	1.74	0.80
21-8	7.4	3.8	16.3	23.6	19.4	3.2	19.2	1.7	1.9	-	-
21-9	7.2	4.0	28.1	11.8	13.5	3.0	22.5	1.9	1.9	2.15	1.05
21-10	7.2	4.2	26.1	13.8	14.6	3.0	22.3	1.7	1.8	1.64	0.82
21-11	7.1	4.2	29.2	11.5	12.7	3.0	22.5	1.8	1.8	2.20	1.09
21-12	7.2	4.1	26.2	13.6	14.2	3.1	22.4	1.8	1.9	1.71	0.80
21-13	7.1	4.3	33.7	7.1	10.0	2.7	24.1	2.0	1.8	3.05	1.47
21-14	7.4	3.7	16.9	21.9	19.6	3.1	19.2	1.8	2.0	0.29	tr
21-15	7.7	3.6	15.6	24.3	20.2	2.9	18.1	1.8	1.8	-	-

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### EXAMPLE 8

#### Fatty acid analysis of Cpal2 transgenic Linola plants

The binary plasmid construct described above comprising the Cpal2 cDNA clone 5 (Figure 9) was transformed into *Agrobacterium tumefaciens* strain AGL1, using electroporation. The transformed *A. tumefaciens* was used to infect *Linum usitatissimum* var. Eyre explants as described by Lawrence *et al* (1989), except that MS media was used as the basal medium for the induction of roots on regenerated shoot material.

Two primary Linola transformants (T0 plants) designated AP20 and AP21 were 10 confirmed as being transgenic by PCR using primers directed against the Cpal2 gene and by showing that these plants were kanamycin resistant. Ten T1 seeds from each plant were analysed individually for fatty acid composition using standard techniques.

As shown in Table 7, seed from AP20 segregated into 3 classes, comprised of three seeds with no vernolic acid, two having greater than 0.7% vernolic acid, and five having 15 intermediate levels (0.13-0.47%) of vernolic acid.

Similarly, seeds from AP21 segregated into 3 classes comprised of five seeds having no vernolic acid, two having greater than 0.25% vernolic acid and three having an intermediate level (0.09-0.14%) of vernolic acid (Table 8).

Thus, a total of twelve seeds were obtained which contained vernolic acid. Eight of 20 the twelve AP20 and AP21 seeds containing vernolic acid also contained 12,13-epoxy-9,15-octadecadienoic acid.

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TABLE 7

Fatty acid composition of 10 individual T1 seeds from  
*Linola Cpal2* primary transformant AP20

T <sub>1</sub> seed	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
1	6.4	3.6	17.8	68.1	2.0	0.2	-	0.6	-	-	-
2	6.0	3.5	25.4	60.8	1.4	0.2	0.2	-	-	0.70	0.23
3	6.0	3.9	20.4	64.6	2.1	0.3	0.6	-	-	-	-
4	6.3	3.5	28.3	57.3	1.3	0.2	0.2	1.4	-	0.34	0.28
5	5.2	4.8	24.9	61.2	1.6	0.3	0.2	0.1	-	0.37	-
6	5.8	4.1	23.3	63.1	1.9	0.2	0.2	0.2	-	0.47	-
7	5.9	4.3	21.7	64.1	2.2	0.2	0.2	0.2	-	0.13	0.12
8	5.9	3.3	22.3	65.2	2.0	0.2	0.2	0.1	0.2	-	-
9	5.6	4.0	25.2	61.4	1.7	0.2	0.2	0.1	-	0.84	-
10	6.2	4.4	27.4	57.9	1.7	0.2	0.2	0.2	-	0.54	-

TABLE 8

Fatty acid composition of 10 individual T1 seeds from  
*Linola Cpal2* primary transformant AP21

T <sub>1</sub> seed	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
1	6.1	4.2	35.2	50.8	1.3	-	-	-	2.0	-	-
2	5.7	5.0	32.9	53.3	1.4	0.2	0.2	0.2	-	0.14	0.21
3	5.9	4.0	35.1	50.8	1.3	0.2	0.2	0.1	1.5	-	-
4	7.5	4.1	38.8	45.5	1.2	0.2	0.3	-	1.7	-	-
5	5.8	5.0	28.8	57.3	1.3	0.2	0.2	0.1	-	0.37	0.06
6	5.8	5.0	44.1	41.4	1.4	0.2	0.2	0.2	-	-	-
7	6.5	4.5	27.9	58.6	1.3	0.2	0.1	0.1	-	-	-
8	6.9	4.6	37.6	48.1	1.2	-	-	-	-	0.10	0.19
9	6.2	4.7	33.7	52.1	1.3	0.2	0.2	0.2	-	0.09	0.07
10	6.1	4.8	29.7	56.6	1.3	0.2	0.2	0.1	-	0.25	0.04

Four T1 plants were established from the kanamycin-resistant seedlings of AP20. All four plants were subsequently shown to produce vernolic acid in their T2 seed (Table 9). Levels of 18:2 epoxy fatty acids were not analysed in these T2 seed.

**TABLE 9**  
**Fatty acid composition of T2 seeds from Linola *Cpal2* T1 progeny of AP20**

T <sub>2</sub> seed	Non-epoxy fatty acids									epoxy fatty acid
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1
A	3.4	3.0	27.4	65.5	0.6	na	na	na	na	0.06
B	3.5	3.1	30.2	62.6	0.6	na	na	na	na	0.07
C	3.6	2.7	33.3	59.8	0.6	na	na	na	na	0.07
D	3.4	3.1	28.2	64.6	0.6	na	na	na	na	0.11

na. = not analysed

### EXAMPLE 9

#### Producing epoxy fatty acids in transgenic organisms

5

Production of an oil rich in vernolic acid was achieved by transforming the epoxygenase gene described herein, in particular SEQ ID NO:1, into *Arabidopsis thaliana*, as described in the preceding Examples. As shown in Table 5, transgenic *A. thaliana* lines expressing SEQ ID NO:1 produce high levels of vernolic acid in their seeds relative to other fatty acids. In particular, in one transgenic line (Cpal-17), the vernolic acid produced is as much as 15.2% (w/w) of total seed fatty acid content.

Production of an oil rich in vernolic acid is also achieved by transforming the epoxygenase gene described herein, in any one of SEQ ID NOs: 1, 3, 5, 19 or 20 and preferably any one of SEQ ID NOs:1 or 3 or 5, into any oil accumulating organism that normally has very high levels of linoleic acid and minimal other competing enzyme activities capable of utilising linoleic acid as a substrate. The genetic sequences of the invention are placed operably under the control of a promoter which produces high-level expression in oilseed, for example the napin seed-specific promoter.

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In one alternative approach to the transformation of *A. thaliana*, high-linoleic genotypes of flax, sunflower, corn or safflower are transformed with the epoxygenase of the invention. High levels of vernolic acid are produced by the transgenic plants during seed oil synthesis, when the epoxygenase gene is expressed at high levels.

5

Alternatively, Linola® (= low linolenic acid) flax is transformed with the epoxygenase of the invention. High levels of vernolic acid are produced by the transgenic Linola® flax plants during seed oil synthesis, when the epoxygenase gene is expressed at high levels.

10 Additionally, the inventors have shown that labelled vernolic acid fed to developing flax seeds is not degraded but is incorporated into storage lipids at all three positions of the triglyceride molecule (see Example 10). Consistent with these data, high levels of vernolic acid synthesised by the introduced epoxygenase are readily deposited into the seed oil triglycerides of this species.

15

### EXAMPLE 10

#### Incorporation of oleic acid and vernolic acid into the lipids of developing linseed cotyledons

20

Detached developing linseed cotyledons (six pairs in each incubation, duplicate incubations) at mid stage of seed development (20 days after flowering) were incubated with 10 nmol of the ammonium salts of either [1-<sup>14</sup>C]vernolic acid (specific activity 3000 d.p.m./nmol) or [1-<sup>14</sup>C]oleic acid (specific activity 5000 d.p.m./nmol) in 0.2 ml phosphate  
25 buffer pH 7.2 for 30 min at 30°C. The cotyledons were then rinsed three times with 1 ml of distilled water and either extracted immediately in an Ultra Turrax according to Bligh and Dyer (1959) or incubated further in 0.5 ml 0.1 M phosphate buffer pH 7.2 for 90 or 270 min before extraction. An aliquot of the lipids in the chloroform phase was methylated and separated on silica gel TLC plates in n-hexane/diethylether/acetic acid (85:15:1). The rest  
30 of the lipids in the chloroform phase of each sample were applied on two separate silica gel

TLC plates and the plates were developed in chloroform/methanol/acetic acid/water (85:15:10:3.5 by vol) for polar lipids separation and in n-hexane/diethylether/acetic acid (60:40:1.5) for neutral lipid separation. Lipid areas with migration corresponding to authentic standards were removed and radioactivity in each lipid were quantified by liquid  
5 scintillation counting.

The recovery of  $^{14}\text{C}$ -label in the chloroform phase is depicted in Figure 12. Somewhat more than half of added radioactivity from both [ $^{14}\text{C}$ ]oleic acid and [ $^{14}\text{C}$ ]vernolic acid was taken up by the cotyledons and recovered as lipophilic substances after the 30 min pulse  
10 labelling. This quantity remained virtually unchanged during the further 270 min of incubation with both substrates. Separation of radioactive methylesters of the lipids showed that most of the radioactivity (92%) from [ $^{14}\text{C}$ ]vernolic acid feeding experiments resided in compounds with the same migration as methyl-vernoleate indicating that the epoxy group remained intact in the linseed cotyledons throughout the 270 min incubation.

15

About 28% of the activity from [ $^{14}\text{C}$ ]vernolic acid feeding which was present in the chloroform phase resided in phosphatidylcholine after 30 min and the radioactivity decreased to only 5% at 300 min of incubation (Figure 13).

20 About 22% of the activity from [ $^{14}\text{C}$ ]oleic acid feeding which was present in the chloroform phase resided in phosphatidylcholine after 30 min and the radioactivity decreased to about 11% at 300 min of incubation (Figure 13).

About 32% of the activity from [ $^{14}\text{C}$ ]vernolic acid feeding which was present in the  
25 chloroform phase resided in triacylglycerols after 30 min and the radioactivity increased to over 60% at 300 min of incubation (Figure 14). The diacylglycerols contained some 24% of the activity in the [ $^{14}\text{C}$ ]vernolic acid feeding experiments and this quantity remained rather constant over the incubation periods.

30 About 5% of the activity from [ $^{14}\text{C}$ ]oleic acid feeding which was present in the

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chloroform phase resided in triacylglycerols after 30 min and the radioactivity increased to 18% at 300 min of incubation (Figure 14). The diacylglycerols contained some 19% of the activity after 30 min in the [ $^{14}\text{C}$ ]oleic acid feeding experiments and this quantity remained rather constant over the incubation periods.

5

The above experiment shows that linseed cotyledons do not metabolise the epoxy group of vernolic acid to any great extent. Further it shows that linseed cotyledons possess mechanisms to efficiently remove vernolic acid from membrane lipids and incorporate them into triacylglycerols.

10

### EXAMPLE 11

#### Cloning of $\Delta 12$ -epoxygenase genes from other epoxy acid containing species

Homologues of the Cpal2  $\Delta 12$ -epoxygenase gene are obtained from other species which are rich in epoxy fatty acids, by cloning the members of the gene family of  $\Delta 12$  mixed function monooxygenases that are highly expressed in developing seeds and comparing their amino acid sequence to those of known  $\Delta 12$ -desaturase and  $\Delta 12$ -epoxygenase sequences. Such genes are cloned either by screening developing seed cDNA libraries with genetic probes based on either the Cpal2 gene (SEQ ID NO:1) or the D12V fragment (SEQ ID NO: 7), or by amplifying PCR fragments using primers designed against conserved sequences of the plant  $\Delta 12$  mixed function monooxygenases, as described herein. Putative  $\Delta 12$ -epoxygenase sequences show greater overall sequence identity to the  $\Delta 12$ -epoxygenase sequences disclosed herein, than to the known  $\Delta 12$ -desaturase sequences.

In one example of this approach, a full-length  $\Delta 12$ -epoxygenase-like sequence was obtained from an unidentified *Crepis sp.* containing high levels of vernolic acid in its seed oils and known not to be *Crepis palaestina*. Poly(A)+ RNA was isolated from developing seeds of this *Crepis sp.* using a QuickPrep Micro mRNA purification kit (Pharmacia Biotechnology) and used to synthesise an oligosaccharide d(T)-primed double-stranded cDNA. The double stranded cDNA thus obtained was then ligated to *EcoR1/ NotI* adaptors (Pharmacia

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Biotechnology) and a cDNA library was constructed using the ZAP-cDNA Gigapack cloning kit (Stratagene). The cDNA library on Hybond N+ membrane filters (Amersham) was screened with the random-labelled D12V fragment (SEQ ID NO: 7) derived from *Crepis alpina* as prescribed by the manufacturer, using standard hybridisation conditions. This  
5 resulted in the purification of a recombinant bacteriophage designated CrepX.

The nucleotide sequence of the CrepX cDNA was determined and is set forth in SEQ ID NO: 3. The deduced amino acid sequence of CrepX (SEQ ID NO: 4) comprises a 374 amino acid protein having 97% identity to the Cpal2  $\Delta$ 12-epoxygenase sequence, but only  
10 57% identity to the *Arabidopsis thaliana* L26296  $\Delta$ 12-desaturase sequence. This clearly demonstrates the presence of a gene in another *Crepis* sp. having high vernolic acid content, which gene is highly homologous to the Cpal2  $\Delta$ 12-epoxygenase gene and is clearly not a desaturase gene.

15 In a second example of this approach, a partial  $\Delta$ 12-epoxygenase-like sequence was obtained from the vernolic acid-containing species *Vernonia galamensis*. First strand cDNA templates were prepared from total RNA isolated from developing seeds of *V. galamensis* using standard procedures.

20 A PCR fragment (550 nucleotides in length), designated as Vgal1, was obtained by amplifying the single-stranded cDNA using primers derived from the deduced amino acid sequence of plant mixed function monooxygenases. The nucleotide sequence of the amplified DNA was determined using standard procedures and is set forth in SEQ ID NO:5.

25 Alignment of the deduced amino acid sequence of the Vgal1 PCR fragment (SEQ ID NO:6) with the full sequence of Cpal2  $\Delta$ 12-epoxygenase and the *Arabidopsis thaliana* L26296  $\Delta$ 12-desaturase (Figure 2) demonstrates that the amplified Vgal1 sequence encodes an amino acid sequence which corresponds to the region spanning amino acid residues 103-285 of the Cpal2 polypeptide. Within this region, the Vgal1 sequence showed greater amino acid  
30 identity with the Cpal2  $\Delta$ 12-epoxygenase sequence (67%) than with the *A. thaliana*

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$\Delta$ 12-desaturase sequence (60%), suggesting that the amplified DNA corresponds to an epoxygenase rather than a desaturase sequence.

5           Those skilled in the art will be aware that the present invention is subject to variations and modifications other than those specifically described herein. It is to be understood that the invention includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more  
10 of said steps or features.

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20

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Commonwealth Scientific and Industrial Research Organisation  
AND Sten Stymne
- (ii) TITLE OF INVENTION: PLANT FATTY ACID EPOXYGENASE GENES AND USES THEREFOR
- 10 (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
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(E) COUNTRY: AUSTRALIA  
(F) ZIP: 3000
- 20 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
25 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:  
(B) FILING DATE:
- 30 (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: AU P06223  
(B) FILING DATE: 15-APR-1997
- (vii) PRIOR APPLICATION DATA:
- 35 (A) APPLICATION NUMBER: AU P06226  
(B) FILING DATE: 15-APR-1997
- (vii) PRIOR APPLICATION DATA:
- 40 (A) APPLICATION NUMBER: US 60/043706  
(B) FILING DATE: 16-APR-1997

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## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/050403

(B) FILING DATE: 20-JUN-1997

## 5 (viii) ATTORNEY/AGENT INFORMATION:

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10 (A) TELEPHONE: +61 3 9254 2777

(B) TELEFAX: +61 3 9254 2770

(C) TELEX: AA 31787

## (2) INFORMATION FOR SEQ ID NO:1:

15

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1358 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

20

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

25

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 30..1151

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGAAGTTGA CCATAATCA TTTATCAAC ATG GGT GCC GGC GGT CGT GGT CGG 53

Met Gly Ala Gly Gly Arg Gly Arg

1

5

35

ACA TCG GAA AAA TCG GTC ATG GAA CGT GTC TCA GTT GAT CCA GTA ACC 101

Thr Ser Glu Lys Ser Val Met Glu Arg Val Ser Val Asp Pro Val Thr

10

15

20

40 TTC TCA CTG AGT GAA TTG AAG CAA GCA ATC CCT CCC CAT TGC TTC CAG 149

- 79 -

Phe Ser Leu Ser Glu Leu Lys Gln Ala Ile Pro Pro His Cys Phe Gln  
 25 30 35 40

AGA TCT GTA ATC CGC TCA TCT TAC TAT GTT GTT CAA GAT CTC ATT ATT 197  
 5 Arg Ser Val Ile Arg Ser Ser Tyr Tyr Val Val Gln Asp Leu Ile Ile  
 45 50 55

GCC TAC ATC TTC TAC TTC CTT GCC AAC ACA TAT ATC CCT ACT CTT CCT 245  
 Ala Tyr Ile Phe Tyr Phe Leu Ala Asn Thr Tyr Ile Pro Thr Leu Pro  
 10 60 65 70

ACT AGT CTA GCC TAC TTA GCT TGG CCC GTT TAC TGG TTC TGT CAA GCT 293  
 Thr Ser Leu Ala Tyr Leu Ala Trp Pro Val Tyr Trp Phe Cys Gln Ala  
 75 80 85

15 AGC GTC CTC ACT GGC TTA TGG ATC CTC GGC CAC GAA TGT GGT CAC CAT 341  
 Ser Val Leu Thr Gly Leu Trp Ile Leu Gly His Glu Cys Gly His His  
 90 95 100

20 GCC TTT AGC AAC TAC ACA TGG TTT GAC GAC ACT GTG GGC TTC ATC CTC 389  
 Ala Phe Ser Asn Tyr Thr Trp Phe Asp Asp Thr Val Gly Phe Ile Leu  
 105 110 115 120

CAC TCA TTT CTC CTC ACC CCG TAT TTC TCT TGG AAA TTC AGT CAC CGG 437  
 25 His Ser Phe Leu Leu Thr Pro Tyr Phe Ser Trp Lys Phe Ser His Arg  
 125 130 135

AAT CAC CAT TCC AAC ACA AGT TCG ATT GAT AAC GAT GAA GTT TAC ATT 485  
 Asn His His Ser Asn Thr Ser Ser Ile Asp Asn Asp Glu Val Tyr Ile  
 30 140 145 150

CCG AAA AGC AAG TCC AAA CTC GCG CGT ATC TAT AAA CTT CTT AAC AAC 533  
 Pro Lys Ser Lys Ser Lys Leu Ala Arg Ile Tyr Lys Leu Leu Asn Asn  
 155 160 165

35 CCA CCT GGT CGG CTG TTG GTT TTG ATT ATC ATG TTC ACC CTA GGA TTT 581  
 Pro Pro Gly Arg Leu Leu Val Leu Ile Ile Met Phe Thr Leu Gly Phe  
 170 175 180

40 CCT TTA TAC CTC TTG ACA AAT ATT TCC GGC AAG AAA TAC GAC AGG TTT 629

- 80 -

Pro Leu Tyr Leu Leu Thr Asn Ile Ser Gly Lys Lys Tyr Asp Arg Phe  
 185 190 195 200

GCC AAC CAC TTC GAC CCC ATG AGT CCA ATT TTC AAA GAA CGT GAG CGG 677  
 5 Ala Asn His Phe Asp Pro Met Ser Pro Ile Phe Lys Glu Arg Glu Arg  
 205 210 215

TTT CAG GTC TTC CTT TCG GAT CTT GGT CTT CTT GCC GTG TTT TAT GGA 725  
 Phe Gln Val Phe Leu Ser Asp Leu Gly Leu Leu Ala Val Phe Tyr Gly  
 10 220 225 230

ATT AAA GTT GCT GTA GCA AAT AAA GGA GCT GCT TGG GTA GCG TGC ATG 773  
 Ile Lys Val Ala Val Ala Asn Lys Gly Ala Ala Trp Val Ala Cys Met  
 235 240 245

15 TAT GGA GTT CCG GTA TTA GGC GTA TTT ACC TTT TTC GAT GTG ATC ACC 821  
 Tyr Gly Val Pro Val Leu Gly Val Phe Thr Phe Phe Asp Val Ile Thr  
 250 255 260

20 TTC TTG CAC CAC ACC CAT CAG TCG TCG CCT CAT TAT GAT TCA ACT GAA 869  
 Phe Leu His His Thr His Gln Ser Ser Pro His Tyr Asp Ser Thr Glu  
 265 270 275 280

TGG AAC TGG ATC AGA GGG GCC TTG TCA GCA ATC GAT AGG GAC TTT GGA 917  
 25 Trp Asn Trp Ile Arg Gly Ala Leu Ser Ala Ile Asp Arg Asp Phe Gly  
 285 290 295

TTC CTG AAT AGT GTT TTC CAT GAT GTT ACA CAC ACT CAT GTC ATG CAT 965  
 Phe Leu Asn Ser Val Phe His Asp Val Thr His Thr His Val Met His  
 30 300 305 310

CAT TTG TTT TCA TAC ATT CCA CAC TAT CAT GCA AAG GAG GCA AGG GAT 1013  
 His Leu Phe Ser Tyr Ile Pro His Tyr His Ala Lys Glu Ala Arg Asp  
 315 320 325

35 GCA ATC AAG CCA ATC TTG GGC GAC TTT TAT ATG ATC GAC AGG ACT CCA 1061  
 Ala Ile Lys Pro Ile Leu Gly Asp Phe Tyr Met Ile Asp Arg Thr Pro  
 330 335 340

40 ATT TTA AAA GCA ATG TGG AGA GAG GGC AGG GAG TGC ATG TAC ATC GAG 1109

- 81 -

Ile Leu Lys Ala Met Trp Arg Glu Gly Arg Glu Cys Met Tyr Ile Glu  
 345 350 355 360

CCT GAT AGC AAG CTC AAA GGT GTT TAT TGG TAT CAT AAA TTG 1151  
 5 Pro Asp Ser Lys Leu Lys Gly Val Tyr Trp Tyr His Lys Leu  
 365 370

TGATCATATG CAAAATGCAC ATGCATTTTC AAACCCTCTA GTTACGTTTG TTCTATGTAT 1211

10 AATAAACCGC CGGTCCTTTG GTTGACTATG CCTAAGCCAG GCGAAACAGT TAAATAATAT 1271

CGGTATGATG TGTAATGAAA GTATGTGGTT GTCTGGTTTT GTTGCTATGA AAGAAAGTAT 1331

GTGGTTGTCG GTCAAAAAAA AAAAAAA 1358  
 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 374 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Ala Gly Gly Arg Gly Arg Thr Ser Glu Lys Ser Val Met Glu  
 1 5 10 15

30 Arg Val Ser Val Asp Pro Val Thr Phe Ser Leu Ser Glu Leu Lys Gln  
 20 25 30

Ala Ile Pro Pro His Cys Phe Gln Arg Ser Val Ile Arg Ser Ser Tyr  
 35 35 40 45

Tyr Val Val Gln Asp Leu Ile Ile Ala Tyr Ile Phe Tyr Phe Leu Ala  
 50 55 60

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	Asn Thr Tyr Ile Pro Thr Leu Pro Thr Ser Leu Ala Tyr Leu Ala Trp	
	65	70 75 80
5	Pro Val Tyr Trp Phe Cys Gln Ala Ser Val Leu Thr Gly Leu Trp Ile	
	85	90 95
	Leu Gly His Glu Cys Gly His His Ala Phe Ser Asn Tyr Thr Trp Phe	
	100	105 110
10	Asp Asp Thr Val Gly Phe Ile Leu His Ser Phe Leu Leu Thr Pro Tyr	
	115	120 125
	Phe Ser Trp Lys Phe Ser His Arg Asn His His Ser Asn Thr Ser Ser	
	130	135 140
15	Ile Asp Asn Asp Glu Val Tyr Ile Pro Lys Ser Lys Ser Lys Leu Ala	
	145	150 155 160
	Arg Ile Tyr Lys Leu Leu Asn Asn Pro Pro Gly Arg Leu Leu Val Leu	
20	165	170 175
	Ile Ile Met Phe Thr Leu Gly Phe Pro Leu Tyr Leu Leu Thr Asn Ile	
	180	185 190
25	Ser Gly Lys Lys Tyr Asp Arg Phe Ala Asn His Phe Asp Pro Met Ser	
	195	200 205
	Pro Ile Phe Lys Glu Arg Glu Arg Phe Gln Val Phe Leu Ser Asp Leu	
	210	215 220
30	Gly Leu Leu Ala Val Phe Tyr Gly Ile Lys Val Ala Val Ala Asn Lys	
	225	230 235 240
	Gly Ala Ala Trp Val Ala Cys Met Tyr Gly Val Pro Val Leu Gly Val	
35	245	250 255
	Phe Thr Phe Phe Asp Val Ile Thr Phe Leu His His Thr His Gln Ser	
	260	265 270

- 83 -

Ser Pro His Tyr Asp Ser Thr Glu Trp Asn Trp Ile Arg Gly Ala Leu  
275 280 285

5 Ser Ala Ile Asp Arg Asp Phe Gly Phe Leu Asn Ser Val Phe His Asp  
290 295 300

Val Thr His Thr His Val Met His His Leu Phe Ser Tyr Ile Pro His  
305 310 315 320

10 Tyr His Ala Lys Glu Ala Arg Asp Ala Ile Lys Pro Ile Leu Gly Asp  
325 330 335

Phe Tyr Met Ile Asp Arg Thr Pro Ile Leu Lys Ala Met Trp Arg Glu  
340 345 350

15 Gly Arg Glu Cys Met Tyr Ile Glu Pro Asp Ser Lys Leu Lys Gly Val  
355 360 365

20 Tyr Trp Tyr His Lys Leu  
370

25 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 1312 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Crepis sp.

(ix) FEATURE:

40 (A) NAME/KEY: CDS  
(B) LOCATION: 26..1147

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

	TGTTGACCAT AAATCATCTA TCAAC ATG GGT GCC GGC GGC CGT GGT CGG ACA	52
	Met Gly Ala Gly Gly Arg Gly Arg Thr	
5	1 5	
	TCG GAA AAG TCG GTC ATG GAA CGT GTC TCA GTT GAT CCA GTA ACC TTC	100
	Ser Glu Lys Ser Val Met Glu Arg Val Ser Val Asp Pro Val Thr Phe	
	10 15 20 25	
10		
	TCA CTG AGT GAT TTG AAG CAA GCA ATC CCT CCA CAT TGC TTC CAG CGA	148
	Ser Leu Ser Asp Leu Lys Gln Ala Ile Pro Pro His Cys Phe Gln Arg	
	30 35 40	
15		
	TCT GTC ATC CGT TCA TCT TAT TAC GTT GTT CAG GAT CTC ATA ATT GCC	196
	Ser Val Ile Arg Ser Ser Tyr Tyr Val Val Gln Asp Leu Ile Ile Ala	
	45 50 55	
	TAC ATC TTC TAC TTC CTT GCC AAC ACA TAT ATC CCT AAT CTC CCT CAT	244
20	Tyr Ile Phe Tyr Phe Leu Ala Asn Thr Tyr Ile Pro Asn Leu Pro His	
	60 65 70	
	CCT CTA GCC TAC TTA GCT TGG CCG CTT TAC TGG TTC TGT CAA GCT AGC	292
	Pro Leu Ala Tyr Leu Ala Trp Pro Leu Tyr Trp Phe Cys Gln Ala Ser	
25	75 80 85	
	GTC CTC ACT GGG TTA TGG ATC CTC GGC CAT GAA TGT GGT CAC CAT GCC	340
	Val Leu Thr Gly Leu Trp Ile Leu Gly His Glu Cys Gly His His Ala	
	90 95 100 105	
30		
	TAT AGC AAC TAC ACA TGG GTT GAC GAC ACT GTG GGC TTC ATC ATC CAT	388
	Tyr Ser Asn Tyr Thr Trp Val Asp Asp Thr Val Gly Phe Ile Ile His	
	110 115 120	
35		
	TCA TTT CTC CTC ACC CCG TAT TTC TCT TGG AAA TAC AGT CAC CGG AAT	436
	Ser Phe Leu Leu Thr Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Asn	
	125 130 135	

- 85 -

	CAC CAT TCC AAC ACA AGT TCG ATT GAT AAC GAT GAA GTT TAC ATT CCG	484
	His His Ser Asn Thr Ser Ser Ile Asp Asn Asp Glu Val Tyr Ile Pro	
	140 145 150	
5	AAA AGC AAG TCC AAA CTC AAG CGT ATC TAT AAA CTT CTT AAC AAC CCA	532
	Lys Ser Lys Ser Lys Leu Lys Arg Ile Tyr Lys Leu Leu Asn Asn Pro	
	155 160 165	
10	CCT GGT CGA CTG TTG GTT TTG GTT ATC ATG TTC ACC CTA GGA TTT CCT	580
	Pro Gly Arg Leu Leu Val Leu Val Ile Met Phe Thr Leu Gly Phe Pro	
	170 175 180 185	
15	TTA TAC CTC TTG ACA AAT ATT TCC GGC AAG AAA TAC GAT AGG TTT GCC	628
	Leu Tyr Leu Leu Thr Asn Ile Ser Gly Lys Lys Tyr Asp Arg Phe Ala	
	190 195 200	
20	AAC CAC TTC GAC CCC ATG AGT CCA ATT TTC AAA GAA CGT GAG CGG TTT	676
	Asn His Phe Asp Pro Met Ser Pro Ile Phe Lys Glu Arg Glu Arg Phe	
	205 210 215	
25	CAG GTC TTC CTT TCG GAT CTT GGT CTT CTT GCT GTG TTT TAT GGA ATT	724
	Gln Val Phe Leu Ser Asp Leu Gly Leu Leu Ala Val Phe Tyr Gly Ile	
	220 225 230	
30	AAA GTT GCT GTA GCA AAT AAA GGA GCT GCT TGG GTG GCG TGC ATG TAT	772
	Lys Val Ala Val Ala Asn Lys Gly Ala Ala Trp Val Ala Cys Met Tyr	
	235 240 245	
35	GGA GTT CCG GTG CTA GGC GTA TTT ACC TTT TTC GAT GTG ATC ACG TTC	820
	Gly Val Pro Val Leu Gly Val Phe Thr Phe Phe Asp Val Ile Thr Phe	
	250 255 260 265	
40	TTA CAC CAC ACC CAT CAG TCG TCG CCT CAT TAT GAT TCA ACT GAA TGG	868
	Leu His His Thr His Gln Ser Ser Pro His Tyr Asp Ser Thr Glu Trp	
	270 275 280	
	AAC TGG ATC AGA GGG GCT TTG TCA GCA ATC GAT AGN GAC TTT GGG TTC	916
	Asn Trp Ile Arg Gly Ala Leu Ser Ala Ile Asp Arg Asp Phe Gly Phe	
	285 290 295	

- 86 -

CTG AAT AGT GTT TTC CAT GAT GTN ACA CAC ACT CAC GTC ATG CAT CAT 964  
 Leu Asn Ser Val Phe His Asp Val Thr His Thr His Val Met His His  
 300 305 310

5 TTG TTT TCA TAC ATT CCA CAC TAT CAT GCA AAG GAA GCA AGG GAT GCA 1012  
 Leu Phe Ser Tyr Ile Pro His Tyr His Ala Lys Glu Ala Arg Asp Ala  
 315 320 325

ATC AAA CCG ATC TTG GGC GAC TTT TAT ATG ATC GAT AGG ACT CCA ATT 1060  
 10 Ile Lys Pro Ile Leu Gly Asp Phe Tyr Met Ile Asp Arg Thr Pro Ile  
 330 335 340 345

TTA AAA GCA ATG TGG AGA GAG GGC AGG GAA TGC ATG TAC ATC GAG CCT 1108  
 Leu Lys Ala Met Trp Arg Glu Gly Arg Glu Cys Met Tyr Ile Glu Pro  
 15 350 355 360

GAT AGC AAG CTC AAA GGT GTT TAT TGG TAT CAT AAA TTG TGATCATATG 1157  
 Asp Ser Lys Leu Lys Gly Val Tyr Trp Tyr His Lys Leu  
 365 370

20 CAAAATGCAC ATGCATTTTC AAACCCTCTA GTTACCTTTG TTCTATGTAT AATAAGACCG 1217

CCGGTCCTAT GGTTTTCTAT GCCTAAGCCA GGCAGAAATAG TTAAATAATA TCGGTATGAT 1277

25 GTAATGAAAG TATGTGGTTG TCTAAAAAAA AAAAA 1312

(2) INFORMATION FOR SEQ ID NO:4:

30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 374 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Ala Gly Gly Arg Gly Arg Thr Ser Glu Lys Ser Val Met Glu  
 40 1 5 10 15

- 87 -

Arg Val Ser Val Asp Pro Val Thr Phe Ser Leu Ser Asp Leu Lys Gln  
 20 25 30

Ala Ile Pro Pro His Cys Phe Gln Arg Ser Val Ile Arg Ser Ser Tyr  
 5 35 40 45

Tyr Val Val Gln Asp Leu Ile Ile Ala Tyr Ile Phe Tyr Phe Leu Ala  
 50 55 60

10 Asn Thr Tyr Ile Pro Asn Leu Pro His Pro Leu Ala Tyr Leu Ala Trp  
 65 70 75 80

Pro Leu Tyr Trp Phe Cys Gln Ala Ser Val Leu Thr Gly Leu Trp Ile  
 85 90 95

15 Leu Gly His Glu Cys Gly His His Ala Tyr Ser Asn Tyr Thr Trp Val  
 100 105 110

Asp Asp Thr Val Gly Phe Ile Ile His Ser Phe Leu Leu Thr Pro Tyr  
 20 115 120 125

Phe Ser Trp Lys Tyr Ser His Arg Asn His His Ser Asn Thr Ser Ser  
 130 135 140

25 Ile Asp Asn Asp Glu Val Tyr Ile Pro Lys Ser Lys Ser Lys Leu Lys  
 145 150 155 160

Arg Ile Tyr Lys Leu Leu Asn Asn Pro Pro Gly Arg Leu Leu Val Leu  
 165 170 175

30 Val Ile Met Phe Thr Leu Gly Phe Pro Leu Tyr Leu Leu Thr Asn Ile  
 180 185 190

Ser Gly Lys Lys Tyr Asp Arg Phe Ala Asn His Phe Asp Pro Met Ser  
 35 195 200 205

Pro Ile Phe Lys Glu Arg Glu Arg Phe Gln Val Phe Leu Ser Asp Leu  
 210 215 220

- 88 -

Gly Leu Leu Ala Val Phe Tyr Gly Ile Lys Val Ala Val Ala Asn Lys  
 225 230 235 240

5 Gly Ala Ala Trp Val Ala Cys Met Tyr Gly Val Pro Val Leu Gly Val  
 245 250 255

Phe Thr Phe Phe Asp Val Ile Thr Phe Leu His His Thr His Gln Ser  
 260 265 270

10 Ser Pro His Tyr Asp Ser Thr Gly Trp Asn Trp Ile Arg Gly Ala Leu  
 275 280 285

Ser Ala Ile Asp Arg Asp Phe Gly Phe Leu Asn Ser Val Phe His Asp  
 15 290 295 300

Val Thr His Thr His Val Met His His Leu Phe Ser Tyr Ile Pro His  
 305 310 315 320

20 Tyr His Ala Lys Glu Ala Arg Asp Ala Ile Lys Pro Ile Leu Gly Asp  
 325 330 335

Phe Tyr Met Ile Asp Arg Thr Pro Ile Leu Lys Ala Met Trp Arg Glu  
 340 345 350

25 Gly Arg Glu Cys Met Tyr Ile Glu Pro Asp Ser Lys Leu Lys Gly Val  
 355 360 365

Tyr Trp Tyr His Lys Leu  
 30 370

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 550 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

(A) ORGANISM: *Vernonia galamensis*

(ix) **FEATURE:**

5

(A) NAME/KEY: CDS

(B) LOCATION: 1..549

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

10

CAT CAC GCC TTC AGT GAC TAT CAA TGG ATA GAC GAC ACT GTG GGC TTC 48  
His His Ala Phe Ser Asp Tyr Gln Trp Ile Asp Asp Thr Val Gly Phe  
1 5 10 15

15 ATC CTT CAC TTT GCA CTC TTC ACC CCT TAT TTC TCT TGG AAA TAC AGT 96  
Ile Leu His Phe Ala Leu Phe Thr Pro Tyr Phe Ser Trp Lys Tyr Ser  
20 25 30

CAC CGT AAT CAC CAT GCC AAC ACA AAC TCT CTT GTA ACC GAT GAA GTA 144  
20 His Arg Asn His His Ala Asn Thr Asn Ser Leu Val Thr Asp Glu Val  
35 40 45

TAC ATC CCT AAA GTT AAA TCC AAG GTC AAG ATT TAT TCC AAA ATC CTT 192  
Tyr Ile Pro Lys Val Lys Ser Lys Val Lys Ile Tyr Ser Lys Ile Leu  
25            50                    55                            60

AAC AAC CCT CCT GGT CGC GTT TTC ACC TTG GCT TTC AGA TTG ATC GTG 240  
Asn Asn Pro Pro Gly Arg Val Phe Thr Leu Ala Phe Arg Leu Ile Val  
65 70 75 80

30

GGT TTT CCT TTA TAC CTT TTC ACC AAT GTT TCA GGC AAG AAA TAC GAA 288  
Gly Phe Pro Leu Tyr Leu Phe Thr Asn Val Ser Gly Lys Lys Tyr Glu  
85 90 95

35 CGT TTT GCC AAC CAT TTT GAT CCC ATG AGT CCC ATT TTC ACC GAG CGT 336  
Arg Phe Ala Asn His Phe Asp Pro Met Ser Pro Ile Phe Thr Glu Arg  
100 105 110

- 90 -

GAG CAT GTA CAA GTC TTG CTT TCT GAT TTT GGT CTC ATA GCA GTT GCT 384  
 Glu His Val Gln Val Leu Leu Ser Asp Phe Gly Leu Ile Ala Val Ala  
 115 120 125

5 TAC GTG GTT CGT CAA GCT GTA CTG GCT AAA GGA GGT GCT TGG GTG ATG 432  
 Tyr Val Val Arg Gln Ala Val Leu Ala Lys Gly Gly Ala Trp Val Met  
 130 135 140

TGC ATT TAC GGA GTT CCT GTG CTG GCC GTA AAC GCA TTC TTT GTT TTA 480  
 10 Cys Ile Tyr Gly Val Pro Val Leu Ala Val Asn Ala Phe Phe Val Leu  
 145 150 155 160

ATC ACT TAT CTT CAC CAC ACG CAT CTC TCA CTG CCC CAC TAT GAT AGC 528  
 Ile Thr Tyr Leu His His Thr His Leu Ser Leu Pro His Tyr Asp Ser  
 15 165 170 175

TCA GAA TGG GAC TGG CTA CGA G 550  
 Ser Glu Trp Asp Trp Leu Arg  
 180

20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 183 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

His His Ala Phe Ser Asp Tyr Gln Trp Ile Asp Asp Thr Val Gly Phe  
 1 5 10 15

35 Ile Leu His Phe Ala Leu Phe Thr Pro Tyr Phe Ser Trp Lys Tyr Ser  
 20 25 30

His Arg Asn His His Ala Asn Thr Asn Ser Leu Val Thr Asp Glu Val  
 40 45

- 91 -

[illegible]

(2) INFORMATION FOR SEQ ID NO:7:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 177 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Crepis alpina*

40

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(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..177

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

	GAA TGC GGT CAC CAT GCC TTC AGC GAC TAC CAG TGG GTT GAC GAC AAT	48
	Glu Cys Gly His His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Asn	
10	1 5 10 15	
	GTG GGC TTC ATC CTC CAC TCG TTT CTC ATG ACC CCG TAT TTC TCC TGG	96
	Val Gly Phe Ile Leu His Ser Phe Leu Met Thr Pro Tyr Phe Ser Trp	
	20 25 30	
15		
	AAA TAC AGC CAC CGG AAC CAC CAT GCC AAC ACA AAT TCG CTT GAC AAC	144
	Lys Tyr Ser His Arg Asn His His Ala Asn Thr Asn Ser Leu Asp Asn	
	35 40 45	
20	GAT GAA GTT TAC ATC CCC AAA AGC AAG GCC AAA	177
	Asp Glu Val Tyr Ile Pro Lys Ser Lys Ala Lys	
	50 55	

25 (2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 59 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

35

Glu Cys Gly His His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Asn  
 1 5 10 15  
 Val Gly Phe Ile Leu His Ser Phe Leu Met Thr Pro Tyr Phe Ser Trp  
 40 20 25 30

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Lys Tyr Ser His Arg Asn His His Ala Asn Thr Asn Ser Leu Asp Asn  
 35 40 45

Asp Glu Val Tyr Ile Pro Lys Ser Lys Ala Lys  
 5 50 55

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 383 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Arabidopsis thaliana

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Gly Ala Gly Gly Arg Met Pro Val Pro Thr Ser Ser Lys Lys Ser  
 1 5 10 15

25

Glu Thr Asp Thr Thr Lys Arg Val Pro Cys Glu Lys Pro Pro Phe Ser  
 20 25 30

30

Val Gly Asp Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser  
 35 40 45

Ile Pro Arg Ser Phe Ser Tyr Leu Ile Ser Asp Ile Ile Ile Ala Ser  
 50 55 60

35

Cys Phe Tyr Tyr Val Ala Thr Asn Tyr Phe Ser Leu Leu Pro Gln Pro  
 65 70 75 80

Leu Ser Tyr Leu Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val  
 85 90 95

40

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Leu Thr Gly Ile Trp Val Ile Ala His Glu Cys Gly His His Ala Phe  
 100 105 110

5 Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser  
 115 120 125

Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His  
 130 135 140

10 His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys  
 145 150 155 160

Gln Lys Ser Ala Ile Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu  
 165 170 175

15 Gly Arg Ile Met Met Leu Thr Val Gln Phe Val Leu Gly Trp Pro Leu  
 180 185 190

Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Phe Ala Cys  
 20 195 200 205

His Phe Phe Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu Gln  
 210 215 220

25 Ile Tyr Leu Ser Asp Ala Gly Ile Leu Ala Val Cys Phe Gly Leu Tyr  
 225 230 235 240

Arg Tyr Ala Ala Ala Gln Gly Met Ala Ser Met Ile Cys Leu Tyr Gly  
 245 250 255

30 Val Pro Leu Leu Ile Val Asn Ala Phe Leu Val Leu Ile Thr Tyr Leu  
 260 265 270

Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp Asp  
 35 275 280 285

Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile Leu  
 290 295 300

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Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His Leu  
305                    310                    315                    320

5 Phe Ser Thr Met Pro His Tyr Asn Ala Met Glu Ala Thr Lys Ala Ile  
325 330 335

Lys Pro Ile Leu Gly Asp Tyr Tyr Gln Phe Asp Gly Thr Pro Trp Tyr  
340 345 350

10 Val Ala Met Tyr Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro Asp  
355 360 365

Arg Glu Gly Asp Lys Lys Gly Val Tyr Trp Tyr Asn Asn Lys Leu  
370 375 380

15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

20 (B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Brassica juncea*

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10;

Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Ser Pro Lys Lys Ser  
1                5                10                15

35      Glu Thr Asp Thr Leu Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr  
                    20                      25                      30

Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser  
35 40 45

40

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	Ile	Pro	Arg	Ser	Phe	Ser	Tyr	Leu	Ile	Trp	Asp	Ile	Ile	Val	Ala	Ser	
	50						55					60					
5	Cys	Phe	Tyr	Tyr	Val	Ala	Thr	Thr	Tyr	Phe	Pro	Leu	Leu	Pro	His	Pro	
	65					70				75					80		
	Leu	Ser	Tyr	Val	Ala	Trp	Pro	Leu	Tyr	Trp	Ala	Cys	Gln	Gly	Val	Val	
					85					90					95		
10	Leu	Thr	Gly	Val	Trp	Val	Ile	Ala	His	Glu	Cys	Gly	His	His	Ala	Phe	
					100					105					110		
	Ser	Asp	Tyr	Gln	Trp	Leu	Asp	Asp	Thr	Val	Gly	Leu	Ile	Phe	His	Ser	
				115					120					125			
15	Phe	Leu	Leu	Val	Pro	Tyr	Phe	Ser	Trp	Lys	Tyr	Ser	His	Arg	Arg	His	
	130							135					140				
	His	Ser	Asn	Thr	Gly	Ser	Leu	Glu	Arg	Asp	Glu	Val	Phe	Val	Pro	Lys	
20	145					150				155					160		
	Lys	Lys	Ser	Asp	Ile	Lys	Trp	Tyr	Gly	Lys	Tyr	Leu	Asn	Asn	Pro	Leu	
					165					170					175		
25	Gly	Arg	Thr	Val	Met	Leu	Thr	Val	Gln	Phe	Thr	Leu	Gly	Trp	Pro	Leu	
					180					185					190		
	Tyr	Trp	Ala	Phe	Asn	Val	Ser	Gly	Arg	Pro	Tyr	Pro	Glu	Gly	Phe	Ala	
					195					200					205		
30	Cys	His	Phe	His	Pro	Asn	Ala	Pro	Ile	Tyr	Asn	Asp	Arg	Glu	Arg	Leu	
	210							215					220				
	Gln	Ile	Tyr	Val	Ser	Asp	Ala	Gly	Ile	Leu	Ala	Val	Cys	Tyr	Gly	Leu	
35	225					230				235					240		
	Tyr	Arg	Tyr	Ala	Ala	Ala	Gln	Gly	Val	Ala	Ser	Met	Val	Cys	Leu	Tyr	
						245				250					255		

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Gly Val Pro Leu Leu Ile Val Asn Ala Phe Leu Val Leu Ile Thr Tyr  
 260 265 270

5 Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp  
 275 280 285

Asp Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile  
 290 295 300

10 Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His  
 305 310 315 320

Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Val Thr Lys Ala  
 325 330 335

15 Ile Lys Pro Ile Leu Gly Asp Tyr Tyr Gln Phe Asp Gly Thr Pro Trp  
 340 345 350

Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro  
 20 355 360 365

Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu  
 370 375 380

25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 383 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

35

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Glycine max

40

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

	Met	Gly	Ala	Gly	Gly	Arg	Thr	Asp	Val	Pro	Pro	Ala	Asn	Arg	Lys	Ser	
5	1				5					10						15	
	Glu	Val	Asp	Pro	Leu	Lys	Arg	Val	Pro	Phe	Glu	Lys	Pro	Gln	Phe	Ser	
				20					25					30			
	Leu	Ser	Gln	Ile	Lys	Lys	Ala	Ile	Pro	Pro	His	Cys	Phe	Gln	Arg	Ser	
10			35					40					45				
	Val	Leu	Arg	Ser	Phe	Ser	Tyr	Val	Val	Tyr	Asp	Leu	Thr	Ile	Ala	Phe	
		50					55					60					
15	Cys	Leu	Tyr	Tyr	Val	Ala	Thr	His	Tyr	Phe	His	Leu	Leu	Pro	Gly	Pro	
	65					70					75					80	
	Leu	Ser	Phe	Arg	Gly	Met	Ala	Ile	Tyr	Trp	Ala	Val	Gln	Gly	Cys	Ile	
				85						90					95		
20	Leu	Thr	Gly	Val	Trp	Val	Ile	Ala	His	Glu	Cys	Gly	His	His	Ala	Phe	
			100						105					110			
	Ser	Asp	Tyr	Gln	Leu	Leu	Asp	Asp	Ile	Val	Gly	Leu	Ile	Leu	His	Ser	
25			115					120					125				
	Ala	Leu	Leu	Val	Pro	Tyr	Phe	Ser	Trp	Lys	Tyr	Ser	His	Arg	Arg	His	
		130					135					140					
30	His	Ser	Asn	Thr	Gly	Ser	Leu	Glu	Arg	Asp	Glu	Val	Phe	Val	Pro	Lys	
	145					150					155					160	
	Gln	Lys	Ser	Cys	Ile	Lys	Trp	Tyr	Ser	Lys	Tyr	Leu	Asn	Asn	Pro	Pro	
				165						170					175		
35	Gly	Arg	Val	Leu	Thr	Leu	Ala	Val	Thr	Leu	Thr	Leu	Gly	Trp	Pro	Leu	
			180						185					190			
	Tyr	Leu	Ala	Leu	Asn	Val	Ser	Gly	Arg	Pro	Tyr	Asp	Arg	Phe	Ala	Cys	
40			195						200				205				

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His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asp Arg Glu Arg Leu Gln  
 210 215 220

5 Ile Tyr Ile Ser Asp Ala Gly Val Leu Ala Val Val Tyr Gly Leu Phe  
 225 230 235 240

Arg Leu Ala Met Ala Lys Gly Leu Ala Trp Val Val Cys Val Tyr Gly  
 245 250 255

10 Val Pro Leu Leu Val Val Asn Gly Phe Leu Val Leu Ile Thr Phe Leu  
 260 265 270

Gln His Thr His Pro Ala Leu Pro His Tyr Thr Ser Ser Glu Trp Asp  
 275 280 285

15 Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile Leu  
 290 295 300

Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His Leu  
 20 305 310 315 320

Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala Ile  
 325 330 335

25 Lys Pro Ile Leu Gly Glu Tyr Tyr Arg Phe Asp Glu Thr Pro Phe Val  
 340 345 350

Lys Ala Met Trp Arg Glu Ala Arg Glu Cys Ile Tyr Val Glu Pro Asp  
 355 360 365

30 Gln Ser Thr Glu Ser Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu  
 370 375 380

## (2) INFORMATION FOR SEQ ID NO:12:

35

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 383 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

40

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Solanum commersonii

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Gly Ala Gly Gly Arg Met Ser Ala Pro Asn Gly Glu Thr Glu Val  
1 5 10 15

Lys Arg Asn Pro Leu Gln Lys Val Pro Thr Ser Lys Pro Pro Phe Thr  
20 25 30

Val Gly Asp Ile Lys Lys Ala Ile Pro Pro His Cys Phe Gln Arg Ser  
15 35 40 45

Leu Ile Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ile Leu Val Ser  
50 55 60

Ile Met Tyr Tyr Val Ala Asn Thr Tyr Phe His Leu Leu Pro Ser Pro  
20 65 70 75 80

Tyr Cys Tyr Ile Ala Trp Pro Ile Tyr Trp Ile Cys Gln Gly Cys Val  
85 90 95

Cys Thr Gly Ile Trp Val Asn Ala His Glu Cys Gly His His Ala Phe  
25 100 105 110

Ser Asp Tyr Gln Trp Val Asp Asp Thr Val Gly Leu Ile Leu His Ser  
30 115 120 125

Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His  
130 135 140

His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys  
35 145 150 155 160

Pro Lys Ser Gln Leu Gly Trp Tyr Ser Lys Tyr Leu Asn Asn Pro Pro  
165 170 175

40

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Gly Arg Val Leu Ser Leu Thr Ile Thr Leu Thr Leu Gly Trp Pro Leu  
 180 185 190

5 Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys  
 195 200 205

His Tyr Asp Pro Tyr Gly Pro Ile Tyr Asn Asn Arg Glu Arg Leu Gln  
 210 215 220

10 Ile Phe Ile Ser Asp Ala Gly Val Leu Gly Val Cys Tyr Leu Leu Tyr  
 225 230 235 240

Arg Ile Ala Leu Val Lys Gly Leu Ala Trp Leu Val Cys Val Tyr Gly  
 245 250 255

15 Val Pro Leu Leu Val Val Asn Gly Phe Leu Val Leu Ile Thr Tyr Leu  
 260 265 270

20 Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Thr Glu Trp Asp  
 275 280 285

Trp Leu Arg Gly Ala Leu Ala Thr Cys Asp Arg Asp Tyr Gly Val Leu  
 290 295 300

25 Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Val His His Leu  
 305 310 315 320

Phe Ser Thr Met Pro His Tyr Asn Ala Met Glu Ala Thr Lys Ala Val  
 325 330 335

30 Lys Pro Leu Leu Gly Asp Tyr Tyr Gln Phe Asp Gly Thr Pro Ile Tyr  
 340 345 350

35 Lys Glu Met Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Lys Asp  
 355 360 365

Glu Ser Ser Gln Gly Lys Gly Val Phe Trp Tyr Lys Asn Lys Leu  
 370 375 380

40

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## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 387 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

10

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: GLycine max

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

15

Met Gly Leu Ala Lys Glu Thr Thr Met Gly Gly Arg Gly Arg Val Ala  
 1 5 10 15

20

Lys Val Glu Val Gln Gly Lys Lys Pro Leu Ser Arg Val Pro Asn Thr  
 20 25 30

Lys Pro Pro Phe Thr Val Gly Gln Leu Lys Lys Ala Ile Pro Pro His  
 35 40 45

25

Cys Phe Gln Arg Ser Leu Leu Thr Ser Phe Ser Tyr Val Val Tyr Asp  
 50 55 60

30

Leu Ser Phe Ala Phe Ile Phe Tyr Ile Ala Thr Thr Tyr Phe His Leu  
 65 70 75 80

Leu Pro Gln Pro Phe Ser Leu Ile Ala Trp Pro Ile Tyr Trp Val Leu  
 85 90 95

35

Gln Gly Cys Leu Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly  
 100 105 110

His His Ala Phe Ser Lys Tyr Gln Trp Val Asp Asp Val Val Gly Leu  
 115 120 125

40

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	Thr	Leu	His	Ser	Thr	Leu	Leu	Val	Pro	Tyr	Phe	Ser	Trp	Lys	Ile	Ser	
	130							135						140			
5	His	Arg	Arg	His	His	Ser	Asn	Thr	Gly	Ser	Leu	Asp	Arg	Asp	Glu	Val	
	145					150					155					160	
	Phe	Val	Pro	Lys	Pro	Lys	Ser	Lys	Val	Ala	Trp	Phe	Ser	Lys	Tyr	Leu	
					165					170						175	
10	Asn	Asn	Pro	Leu	Gly	Arg	Ala	Val	Ser	Leu	Leu	Val	Thr	Leu	Thr	Ile	
				180					185						190		
	Gly	Trp	Pro	Met	Tyr	Leu	Ala	Phe	Asn	Val	Ser	Gly	Arg	Pro	Tyr	Asp	
15				195					200					205			
	Ser	Phe	Ala	Ser	His	Tyr	His	Pro	Tyr	Ala	Pro	Ile	Tyr	Ser	Asn	Arg	
		210					215						220				
20	Glu	Arg	Leu	Leu	Ile	Tyr	Val	Ser	Asp	Val	Ala	Leu	Phe	Ser	Val	Thr	
	225				230						235					240	
	Tyr	Ser	Leu	Tyr	Arg	Val	Ala	Thr	Leu	Lys	Gly	Leu	Val	Trp	Leu	Leu	
					245					250						255	
25	Cys	Val	Tyr	Gly	Val	Pro	Leu	Leu	Ile	Val	Asn	Gly	Phe	Leu	Val	Thr	
				260						265					270		
	Ile	Thr	Tyr	Leu	Gln	His	Thr	His	Phe	Ala	Leu	Pro	His	Tyr	Asp	Ser	
30				275					280					285			
	Ser	Glu	Trp	Asp	Trp	Leu	Lys	Gly	Ala	Leu	Ala	Thr	Met	Asp	Arg	Asp	
		290					295					300					
35	Tyr	Gly	Ile	Leu	Asn	Lys	Val	Phe	His	His	Ile	Thr	Asp	Thr	His	Val	
	305					310					315					320	
	Ala	His	His	Leu	Phe	Ser	Thr	Met	Pro	His	Tyr	His	Ala	Met	Glu	Ala	
					325						330					335	

40

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Thr Asn Ala Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Phe Asp Asp  
 340 345 350

5 Thr Pro Phe Tyr Lys Ala Leu Trp Arg Glu Ala Arg Glu Cys Leu Tyr  
 355 360 365

Val Glu Pro Asp Glu Gly Thr Ser Glu Lys Gly Val Tyr Trp Tyr Arg  
 370 375 380

10 Asn Lys Tyr  
 385

(2) INFORMATION FOR SEQ ID NO:14:

15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 387 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Ricinus communis

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

30 Met Gly Gly Gly Gly Arg Met Ser Thr Val Ile Thr Ser Asn Asn Ser  
 1 5 10 15

Glu Lys Lys Gly Gly Ser Ser His Leu Lys Arg Ala Pro His Thr Lys  
 20 25 30

35 Pro Pro Phe Thr Leu Gly Asp Leu Lys Arg Ala Ile Pro Pro His Cys  
 35 40 45

Phe Glu Arg Ser Phe Val Arg Ser Phe Ser Tyr Val Ala Tyr Asp Val  
 50 55 60

40

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	Cys	Leu	Ser	Phe	Leu	Phe	Tyr	Ser	Ile	Ala	Thr	Asn	Phe	Phe	Pro	Tyr	65	70	75	80
5	Ile	Ser	Ser	Pro	Leu	Ser	Tyr	Val	Ala	Trp	Leu	Val	Tyr	Trp	Leu	Phe	85	90	95	
	Gln	Gly	Cys	Ile	Leu	Thr	Gly	Leu	Trp	Val	Ile	Gly	His	Glu	Cys	Gly	100	105	110	
10	His	His	Ala	Phe	Ser	Glu	Tyr	Gln	Leu	Ala	Asp	Asp	Ile	Val	Gly	Leu	115	120	125	
	Ile	Val	His	Ser	Ala	Leu	Leu	Val	Pro	Tyr	Phe	Ser	Trp	Lys	Tyr	Ser	130	135	140	
15	His	Arg	Arg	His	His	Ser	Asn	Ile	Gly	Ser	Leu	Glu	Arg	Asp	Glu	Val	145	150	155	160
	Phe	Val	Pro	Lys	Ser	Lys	Ser	Lys	Ile	Ser	Trp	Tyr	Ser	Lys	Tyr	Ser	165	170	175	
	Asn	Asn	Pro	Pro	Gly	Arg	Val	Leu	Thr	Leu	Ala	Ala	Thr	Leu	Leu	Leu	180	185	190	
25	Gly	Trp	Pro	Leu	Tyr	Leu	Ala	Phe	Asn	Val	Ser	Gly	Arg	Pro	Tyr	Asp	195	200	205	
	Arg	Phe	Ala	Cys	His	Tyr	Asp	Pro	Tyr	Gly	Pro	Ile	Phe	Ser	Glu	Arg	210	215	220	
30	Glu	Arg	Leu	Gln	Ile	Tyr	Ile	Ala	Asp	Leu	Gly	Ile	Phe	Ala	Thr	Thr	225	230	235	240
	Phe	Val	Leu	Tyr	Gln	Ala	Thr	Met	Ala	Lys	Gly	Leu	Ala	Trp	Val	Met	245	250	255	
35	Arg	Ile	Tyr	Gly	Val	Pro	Leu	Leu	Ile	Val	Asn	Cys	Phe	Leu	Val	Met	260	265	270	

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	Ile Thr Tyr Leu Gln His Thr His Pro Ala Ile Pro Arg Tyr Gly Ser
	275 280 285
5	Ser Glu Trp Asp Trp Leu Arg Gly Ala Met Val Thr Val Asp Arg Asp
	290 295 300
	Tyr Gly Val Leu Asn Lys Val Phe His Asn Ile Ala Asp Thr His Val
	305 310 315 320
10	Ala His His Leu Phe Ala Thr Val Pro His Tyr His Ala Met Glu Ala
	325 330 335
	Thr Lys Ala Ile Lys Pro Ile Met Gly Glu Tyr Tyr Arg Tyr Asp Gly
	340 345 350
15	Thr Pro Phe Tyr Lys Ala Leu Trp Arg Glu Ala Lys Glu Cys Leu Phe
	355 360 365
	Val Glu Pro Asp Glu Gly Ala Pro Thr Gln Gly Val Phe Trp Tyr Arg
20	370 375 380
	Asn Lys Tyr
	385

## 25 (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- 30 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35 (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Glu Cys Gly His His

40 1 5

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## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

5

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

10

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

15

His Arg Asn His His  
1 5

## (2) INFORMATION FOR SEQ ID NO:17:

20

## (i) SEQUENCE CHARACTERISTICS:

25

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (v) FRAGMENT TYPE: internal

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Val Met His His  
1 5

35

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 29 base pairs

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGGAATTCCY TBMGNNNNYT SGGNHTBGG

29

10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 1610 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Euphorbia lagascae

25 (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 8..1546

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGTAACA ATG AAC ACT AAG GAG AAG AAG AAG AAC AGG GTT TCT AAC

49

Met Asn Thr Lys Glu Lys Lys Lys Lys Asn Arg Val Ser Asn

1

5

10

35

ATG TCT ATT CTT CTT TGC TTC CTT TGC CTT CTT CCA GTT TTC CTT GTT

97

Met Ser Ile Leu Leu Cys Phe Leu Cys Leu Leu Pro Val Phe Leu Val

15

20

25

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TCT CTT TCT ATT CTT TCT AAG AGG CTT AAG CCA TCT AAG TGG AAG CTT 145  
 Ser Leu Ser Ile Leu Ser Lys Arg Leu Lys Pro Ser Lys Trp Lys Leu  
 35 40 45

5 CCA CCA GGA CCA AAG ACT CTT CCA ATT ATT GGA AAC CTT CAA GAT GAG 193  
 Pro Pro Gly Pro Lys Thr Leu Pro Ile Ile Gly Asn Leu Gln Asp Glu  
 50 55 60

AGG CAA GAT CCA GAG GCT TCT CTT TCT CAA GGA CAT ATT GCT AGG GGA 241  
 10 Arg Gln Asp Pro Glu Ala Ser Leu Ser Gln Gly His Ile Ala Arg Gly  
 65 70 75

CCA GTT GTT CAT TGC GAG AAG CTT GAG TCT TTC GGA ACT CAA CCA ACT 289  
 Pro Val Val His Cys Glu Lys Leu Glu Ser Phe Gly Thr Gln Pro Thr  
 15 80 85 90

ATT AAG GTT GGA CAT TAT GAT AAG AAC TGC GCT CTT CTT CAT GGA GCT 337  
 Ile Lys Val Gly His Tyr Asp Lys Asn Cys Ala Leu Leu His Gly Ala  
 95 100 105 110

20 GGA GAT GAG CTT CTT GGA AAG CCA TCT CCA CCA AAC GAT GCT TGG GAT 385  
 Gly Asp Glu Leu Leu Gly Lys Pro Ser Pro Pro Asn Asp Ala Trp Asp  
 115 120 125

25 ACT GGA GGA TAT GGA CTT GAG AGG TCT AAG AAC GAG AGG TGG AAG GAG 433  
 Thr Gly Gly Tyr Gly Leu Glu Arg Ser Lys Asn Glu Arg Trp Lys Glu  
 130 135 140

AAG GAG ACT TGG TCT GCT TTC AGG CAA TAT AGG ACT CTT AGG GCT TTC 481  
 30 Lys Glu Thr Trp Ser Ala Phe Arg Gln Tyr Arg Thr Leu Arg Ala Phe  
 145 150 155

GGA ATG GGA GGA AGG TCT TTC GAG CTT ATG AGG TGG CAA GAG GCT CAT 529  
 Gly Met Gly Gly Arg Ser Phe Glu Leu Met Arg Trp Gln Glu Ala His  
 35 160 165 170

TGC CTT GTT GAT GGA TAT GTT TCT AGG AAG GCT TCT GGA ACT GAT CCA 577  
 Cys Leu Val Asp Gly Tyr Val Ser Arg Lys Ala Ser Gly Thr Asp Pro  
 175 180 185 190

40

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ACT AAG GAT CTT GAG GAT TCT AGG TTC AAC ATT ATT ATG GGA GCT ACT 625  
 Thr Lys Asp Leu Glu Asp Ser Arg Phe Asn Ile Ile Met Gly Ala Thr  
 195 200 205

5 TTC AAC CAA GGA CTT GAT TAT AAG ATT AAG ACT TTC CTT GAT AGG CAT 673  
 Phe Asn Gln Gly Leu Asp Tyr Lys Ile Lys Thr Phe Leu Asp Arg His  
 210 215 220

GAG AGG AGG AAC TTC CAA TTC AAC AAC GTT GAT GCT GTT TAT CAT CAA 721  
 10 Glu Arg Arg Asn Phe Gln Phe Asn Asn Val Asp Ala Val Tyr His Gln  
 225 230 235

ATG AAG GAT GCT GAG AGG GGA TTC GTT GAT TCT AGG GGA TGG CAA GAT 769  
 Met Lys Asp Ala Glu Arg Gly Phe Val Asp Ser Arg Gly Trp Gln Asp  
 15 240 245 250

GAG TTC GGA ATT GCT CTT CAA CAA GTT GTT GCT CAA ATT CTT GAT AAG 817  
 Glu Phe Gly Ile Ala Leu Gln Gln Val Val Ala Gln Ile Leu Asp Lys  
 255 260 265 270

20 CCA CTT GAT CAT CAA AAG GCT CTT GAG AGG TGG CAA CCA AGG GAT TCT 865  
 Pro Leu Asp His Gln Lys Ala Leu Glu Arg Trp Gln Pro Arg Asp Ser  
 275 280 285

CTT AAC CAT TTC ATT GGA GCT AGG GAT GAT GAG ATG GTT CAA ATT AAG 913  
 Leu Asn His Phe Ile Gly Ala Arg Asp Asp Glu Met Val Gln Ile Lys  
 290 295 300

TAT GAT TTC TGC AAG GAT GCT CTT AGG ATG TTC GAT ACT GGA ATT CTT 961  
 30 Tyr Asp Phe Cys Lys Asp Ala Leu Arg Met Phe Asp Thr Gly Ile Leu  
 305 310 315

GCT GCT GAT CTT CAA TCT TCT ACT TCT TCT ATT AGG TGG GAG CCA ATT 1009  
 Ala Ala Asp Leu Gln Ser Ser Thr Ser Ser Ile Arg Trp Glu Pro Ile  
 35 320 325 330

GTT GTT ATG CTT CAA GCT GAG GTT AAG GGA GAG ATT TGC GAG GAG CTT 1057  
 Val Val Met Leu Gln Ala Glu Val Lys Gly Glu Ile Cys Glu Glu Leu  
 335 340 345 350

40

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	GAT AGG GTT ATT GCT AGG CAT CAA AGG CCA TCT ATG AAG GAT AAG ATG	1105
	Asp Arg Val Ile Ala Arg His Gln Arg Pro Ser Met Lys Asp Lys Met	
	355 360 365	
5	GTT AAG AGG TAT ACT GCT GCT GTT GTT TGC GAG CTT GAT AGG TAT GCT	1153
	Val Lys Arg Tyr Thr Ala Ala Val Val Cys Glu Leu Asp Arg Tyr Ala	
	370 375 380	
	AAG CTT CTT CCA TCT TCT CTT AGG TGC GTT GCT GCT GAT GAG TGG AAG	1201
10	Lys Leu Leu Pro Ser Ser Leu Arg Cys Val Ala Ala Asp Glu Trp Lys	
	385 390 395	
	TTC AGG GAG TAT CTT ATT CCA GTT GGA ATG ACT GTT GGA AAC CTT AAG	1249
	Phe Arg Glu Tyr Leu Ile Pro Val Gly Met Thr Val Gly Asn Leu Lys	
15	400 405 410	
	ACT ACT GTT ATG CTT GAT CAA AAG GAT CCA GTT GAT CCA GAG CTT TTC	1297
	Thr Thr Val Met Leu Asp Gln Lys Asp Pro Val Asp Pro Glu Leu Phe	
	415 420 425 430	
20		
	GAT GGA ATG TAT GGA CTT GAT GCT GAG GTT CAT TTC GAT AAG ACT GAT	1345
	Asp Gly Met Tyr Gly Leu Asp Ala Glu Val His Phe Asp Lys Thr Asp	
	435 440 445	
25	AGG TTC ATG CCA CCA TTC TCT GCT GGG AGG ATT GCC TGC GCT GGA CAA	1393
	Arg Phe Met Pro Pro Phe Ser Ala Gly Arg Ile Ala Cys Ala Gly Gln	
	450 455 460	
	CTT CTT GCT GCT TAT GAG CTT TTC CTT TTC TTC TGG ACT ATT GCT GAT	1441
30	Leu Leu Ala Ala Tyr Glu Leu Phe Leu Phe Phe Trp Thr Ile Ala Asp	
	465 470 475	
	GTT TTC CAA ATT TTC TCT CTT GCT CAA TTC AAG GAG GGA CAT TGC ACT	1489
	Val Phe Gln Ile Phe Ser Leu Ala Gln Phe Lys Glu Gly His Cys Thr	
35	480 485 490	
	GCT GTT ACT CTT ATT ATT GAT TGC CTT GCT GTT AGG TAT GAT CTT TGC	1537
	Ala Val Thr Leu Ile Ile Asp Cys Leu Ala Val Arg Tyr Asp Leu Cys	
	495 500 505 510	

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CTT GCT AGG TAGGGACCTT TACCGTTTGT GTGACCGTGT CAATGCTTGC 1586  
 Leu Ala Arg

5 AATGGGCTTT TAATAATATT ATTA 1610

(2) INFORMATION FOR SEQ ID NO:20:

10 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1698 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA

20 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 8..1504

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAGAACA ATG GCA CAA TTC GGC ACG AGG GAA ATT CTA GTC TCA CTC TTT 49  
 Met Ala Gln Phe Gly Thr Arg Glu Ile Leu Val Ser Leu Phe  
 1 5 10

30 CTC TTT CTA ATA CTA ATA AAG TTC ACA TTT TTA AAA CTC AAA ACC CCC 97  
 Leu Phe Leu Ile Leu Ile Lys Phe Thr Phe Leu Lys Leu Lys Thr Pro  
 15 20 25 30

35 CAA AAC CTC CCC CCA TCA CCA CCA TCT TTT CCA ATC ACC GGC CAT CTC 145  
 Gln Asn Leu Pro Pro Ser Pro Pro Ser Phe Pro Ile Thr Gly His Leu  
 35 40 45

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	CAT CTC CTA AAA CAA CCA ATC CAC AGA ACT CTC CAC CAA ATC GCC ACC	193
	His Leu Leu Lys Gln Pro Ile His Arg Thr Leu His Gln Ile Ala Thr	
	50 55 60	
5	AAG TAC GGG GAC ATC TTA TTC CTC CGA TTC GGA ACA CGA AAA GTC CTA	241
	Lys Tyr Gly Asp Ile Leu Phe Leu Arg Phe Gly Thr Arg Lys Val Leu	
	65 70 75	
	GTC ATC TCC TCT CTC CCC GCC GTA CAA GAA TGT TTC ACT ATA AAC GAC	289
10	Val Ile Ser Ser Leu Pro Ala Val Gln Glu Cys Phe Thr Ile Asn Asp	
	80 85 90	
	ATC ATT TTC GCT AAC CGC CCA ACA ATT CTC GCC GGG AAG CAC CTC AAT	337
	Ile Ile Phe Ala Asn Arg Pro Thr Ile Leu Ala Gly Lys His Leu Asn	
15	95 100 105 110	
	TAC AAT TCC ACC ACC ATG GGA TTC GCC TCC TAT GGC GAT CAC TGG CGT	385
	Tyr Asn Ser Thr Thr Met Gly Phe Ala Ser Tyr Gly Asp His Trp Arg	
20	115 120 125	
	CAT CTC CGA CGA CTC ACA ACA ATT GAG CTC TTC TCT GCA AAT CGT GTT	433
	His Leu Arg Arg Leu Thr Thr Ile Glu Leu Phe Ser Ala Asn Arg Val	
	130 135 140	
25		
	GCC ATG TTT TCC GGG TTC CGG GCC GAT GAA AGT ACA GCT TTT TAT CAA	481
	Ala Met Phe Ser Gly Phe Arg Ala Asp Glu Ser Thr Ala Phe Tyr Gln	
	145 150 155	
30	ACA GTT GTT CCA GGA AAT CGG GAT TCG GGA AAG ATA GTA ACT TTG ACA	529
	Thr Val Val Pro Gly Asn Arg Asp Ser Gly Lys Ile Val Thr Leu Thr	
	160 165 170	
	TCG AAA CTG ATG GAG CTT ACA CTG AAT AAC ATA ATG AGA ATG GCT GCC	577
35	Ser Lys Leu Met Glu Leu Thr Leu Asn Asn Ile Met Arg Met Ala Ala	
	175 180 185 190	
	GGA AAA CGG TTT TAC GGG AAA GAA GTG AAG GAT GAA GAA GGT GAG TTG	625
	Gly Lys Arg Phe Tyr Gly Lys Glu Val Lys Asp Glu Glu Gly Glu Leu	
40	195 200 205	

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	TTG CAG GAT CTT ATG AAG AAA ATG GAG GCG CTC CGG GGG AAT TCA ACG	673
	Leu Gln Asp Leu Met Lys Lys Met Glu Ala Leu Arg Gly Asn Ser Thr	
	210 215 220	
5	GTG AAA CGA GAT TAT TTT CCA GTA TTG CAG TGG ATT GAT TAT CAG GGA	721
	Val Lys Arg Asp Tyr Phe Pro Val Leu Gln Trp Ile Asp Tyr Gln Gly	
	225 230 235	
	GTA AAG AAG AAG ATG AGG AAC CTG ATG AAG AAA ATG GAC GGG TTC TTG	769
10	Val Lys Lys Lys Met Arg Asn Leu Met Lys Lys Met Asp Gly Phe Leu	
	240 245 250	
	CAA AAT CTC ATT GAT GAA CAC CGA AAC ACG ACG TTG TGG ATC AAT CAA	817
	Gln Asn Leu Ile Asp Glu His Arg Asn Thr Thr Leu Trp Ile Asn Gln	
15	255 260 265 270	
	GTT CGA GCA ACT CGG ACA AAA AGA GGA ACT TGG ACA CTG GTA GAT GTT	865
	Val Arg Ala Thr Arg Thr Lys Arg Gly Thr Trp Thr Leu Val Asp Val	
20	275 280 285	
	ATG TTG AAT CTT AAA AAG ACA CAA CCT GAC TTC TAC ACT GAT CTA ACT	913
	Met Leu Asn Leu Lys Lys Thr Gln Pro Asp Phe Tyr Thr Asp Leu Thr	
	290 295 300	
25		
	ATC AAA GGT GTC ATT CAG ACA ACA CTG ACT GCA GGA TCT CAA ACG TCA	961
	Ile Lys Gly Val Ile Gln Thr Thr Leu Thr Ala Gly Ser Gln Thr Ser	
	305 310 315	
30	GCA GTT ACA CTA GAA TGG GCG CTG TCA CTT CTT CTC AAC CAT CCT CAA	1009
	Ala Val Thr Leu Glu Trp Ala Leu Ser Leu Leu Leu Asn His Pro Gln	
	320 325 330	
	GTA ATG CAC AAA GCT TAT GCC GAA ATA GAG GCG ATT GTC GGG ACC AAC	1057
35	Val Met His Lys Ala Tyr Ala Glu Ile Glu Ala Ile Val Gly Thr Asn	
	335 340 345 350	
	CGC TTA TTA AAC GAA GCC GAC TTA CCA CAT CTA AGC TAT TTA CAA AAC	1105
	Arg Leu Leu Asn Glu Ala Asp Leu Pro His Leu Ser Tyr Leu Gln Asn	
40	355 360 365	

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	ATA ATC ACC GAG ACA TTT CGA CTC TTC CCA CCA GTA CCA CTT TTA CTA	1153
	Ile Ile Thr Glu Thr Phe Arg Leu Phe Pro Pro Val Pro Leu Leu Leu	
	370 375 380	
5	CCC CAT AAA TCA TCA GCA GAT TGC ATA GTT TCC GGG TTT CAC ATA CCA	1201
	Pro His Lys Ser Ser Ala Asp Cys Ile Val Ser Gly Phe His Ile Pro	
	385 390 395	
	CGG GGC ACA ATG TTG CTA GTG AAC ACA TGG AGC ATG AAT AGA AAT CCA	1249
10	Arg Gly Thr Met Leu Leu Val Asn Thr Trp Ser Met Asn Arg Asn Pro	
	400 405 410	
	AGA TTA TGG AAG GAA CCA GAG AAA TTC ATA CCA GAA AGA TTT GAA GGA	1297
15	Arg Leu Trp Lys Glu Pro Glu Lys Phe Ile Pro Glu Arg Phe Glu Gly	
	415 420 425 430	
	GGA GAA AAT ACT GAA GGG TGT AAC TAT AAA TTG CTT CCT TTC GGT GCA	1345
	Gly Glu Asn Thr Glu Gly Cys Asn Tyr Lys Leu Leu Pro Phe Gly Ala	
20	435 440 445	
	GGA AGG CGG GCT TGT CCG GGG GCC GGT GTG GCG AAA CGA ATG GTA GGA	1393
	Gly Arg Arg Ala Cys Pro Gly Ala Gly Val Ala Lys Arg Met Val Gly	
	450 455 460	
25	CTC ACT TTA GGT GCA TTG ATT CAG TGT TTT GAG TGG GAA AGA ATT GGG	1441
	Leu Thr Leu Gly Ala Leu Ile Gln Cys Phe Glu Trp Glu Arg Ile Gly	
	465 470 4 5	
30	GAA GAA GAA ATA GAT TTG AGT GAA GGA ACA GGT CTT ACT ATG CCA AAA	1489
	Glu Glu Glu Ile Asp Leu Ser Glu Gly Thr Gly Leu Thr Met Pro Lys	
	480 485 490	
	GAT TTC CTT TGG AAG TAATATGCAA ACCTCGGCAA AACATGATTA ACTTCTTTTC	1544
35	Asp Phe Leu Trp Lys	
	495	
	TACATTGTTA TAAAAGGTGG GTTCTTTTGC AGGTGCCAAC CCTAATTCAA ATATCGCATT	1604
40	TTTCCCTGC AACCCAGCTG CTAACCAAAT ATCACTGTTT CTCATTATTC CTTATATAAA	1664

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ACCTTAAAGC ACTATTGCG TCCTAAAAAA AAAA

1698

5

## CLAIMS:

1. An isolated nucleic acid molecule which encodes or is complementary to an isolated nucleic acid molecule which encodes a fatty acid epoxxygenase enzyme other than a mammalian arachidonic acid epoxxygenase enzyme.
2. The isolated nucleic acid molecule according to claim 1 wherein the epoxxygenase is a mixed-function monooxygenase enzyme which is capable of catalysing the epoxxygenation of a carbon bond in a fatty acid molecule.
3. The isolated nucleic acid molecule according to claim 2, wherein the carbon bond is a double bond in an unsaturated fatty acid molecule.
4. The isolated nucleic acid molecule according to any one of claims 1 to 3 wherein the epoxxygenase is a  $\Delta 6$ -epoxxygenase enzyme, a  $\Delta 9$ -epoxxygenase enzyme, a  $\Delta 12$ -epoxxygenase or a  $\Delta 15$ -epoxxygenase enzyme.
5. The isolated nucleic acid molecule according to claim 4, wherein the epoxxygenase is a  $\Delta 12$ -epoxxygenase enzyme.
6. The isolated nucleic acid molecule according to any one of claims 1 to 5, derived from a plant.
7. The isolated nucleic acid molecule according to claim 6, wherein the plant is selected from the list comprising *Crepis spp.*, *Euphorbia spp.*, *Chrysanthemum spp.* and *Vernonia spp.*
8. The isolated nucleic acid molecule according to claim 6, wherein the plant produces high levels of vernolic acid.

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9. The isolated nucleic acid molecule according to claim 7, wherein the plant is a *Crepis* sp. selected from the list comprising *Crepis biennis*, *Crepis aurea*, *Crepis conyzaefolia*, *Crepis intermedia*, *Crepis occidentalis*, *Crepis palaestina*, *Crepis vesicaria* and *Crepis xacintha*.
10. The isolated nucleic acid molecule according to claims 8 or 9, wherein the plant is *Crepis palaestina*.
11. The isolated nucleic acid molecule according to claim 7, wherein the plant is *Vernonia galamensis*.
12. The isolated nucleic acid molecule according to any one of claims 1 to 11, comprising a nucleotide sequence which is at least about 65% identical to any one of SEQ ID NOs: 1 or 3 or 5 or a complementary sequence thereto or a homologue, analogue or derivative thereof.
13. The isolated nucleic acid molecule according to any one of claims 1 to 12 capable of hybridizing under at least low stringency conditions to at least 20 contiguous nucleotides contained within any one of SEQ ID NOs: 1 or 3 or 5 or a complementary sequence thereto.
14. An isolated nucleic acid molecule which comprises a nucleotide sequence which is at least 65% identical to any one of SEQ ID NOs: 1 or 3 or 5 or a complementary nucleotide sequence thereto.
15. The isolated nucleic acid molecule according to claim 14, comprising the nucleotide sequence set forth in SEQ ID NO: 1 or at least about 20 contiguous nucleotides thereof.
16. The isolated nucleic acid molecule according to claim 14, comprising the nucleotide sequence set forth in SEQ ID NO: 3 or at least about 20 contiguous nucleotides thereof.

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17. The isolated nucleic acid molecule according to claim 14, comprising the nucleotide sequence set forth in SEQ ID NO: 5 or at least about 20 contiguous nucleotides thereof.

18. An isolated nucleic acid molecule which comprises a nucleotide sequence which is at least 75% identical to at least 200 contiguous nucleotides in any one of SEQ ID NOs: 19 or 20 or a complementary sequence thereto.

19. A genetic construct which comprises the isolated nucleic acid molecule according to any one of claims 1 to 17 operably connected to a promoter sequence, wherein said nucleic acid molecule is capable of being transcribed in the sense or antisense orientation relative to the direction of *in vivo* transcription of a naturally-occurring epoxygenase gene.

20. A method of altering the level of epoxy fatty acids in a cell, tissue, organ or organism, said method comprising introducing a sense, antisense, ribozyme or co-suppression molecule comprising the isolated nucleic acid molecule according to any one of claims 1 to 17 to a cell, tissue, organ or organism and incubating said cell for a time and under conditions sufficient for expression of said sense, antisense, ribozyme or co-suppression molecule to occur.

21. The method according to claim 20, wherein the step of introducing the sense, antisense, ribozyme or co-suppression molecule comprises stably transforming the cell, tissue, organ or organism with the sense, antisense, ribozyme or co-suppression molecule.

22. A method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising culturing a cell which comprises the isolated nucleic acid molecule according to any one of claims 1 to 17 for a time and under conditions sufficient for expression to occur.

23. The method according to claim 22 comprising the additional first step of transforming the cell with the isolated nucleic acid molecule.

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24. A method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising the steps of:

- (i) producing a genetic construct which comprises the isolated nucleic acid molecule according to any one of claims 1 to 17 placed operably under the control of a promoter capable of conferring expression on said genetic sequence in said cell, and optionally an expression enhancer element;
- (ii) transforming said genetic construct into said cell; and
- (iii) selecting transformants which express a functional epoxygenase encoded by the genetic sequence at a high level.

25. A method of producing a recombinant enzymatically active epoxygenase polypeptide in a transgenic plant comprising the steps of:

- (i) producing a genetic construct which comprises the isolated nucleic acid molecule according to any one of claims 1 to 17 placed operably under the control of a seed-specific promoter and optionally an expression enhancer element, wherein said genetic sequences is also placed upstream of a transcription terminator sequence;
- (ii) transforming said genetic construct into a cell or tissue of said plant; and
- (iii) selecting transformants which express a functional epoxygenase encoded by the genetic sequence at a high level in seeds.

26. The method according to claim 25, wherein the plant is an oilseed species that normally produces high levels of linoleic acid.

27. The method according to claims 25 or 26, wherein the plant is selected from the list comprising Linola® flax, oilseed rape, sunflower, safflower, soybean, linseed, sesame, cottonseed, peanut, olive or oil palm, amongst others.

28. A recombinant polypeptide produced according to the method according to any one of claims 22 to 27.

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29. A recombinant polypeptide which comprises a sequence of amino acids set forth in any one of SEQ ID NOs: 2 or 4 or 6 or a homologue, analogue or derivative thereof which is at least about 50% identical thereto.
30. A recombinant polypeptide which is a fusion polypeptide between a part of the amino acid sequence set forth in any one of SEQ ID NOs: 2 or 4 or 6 and an amino acid sequence which is derived from a different mixed function monooxygenase enzyme.
31. The recombinant polypeptide according to claim 30, wherein the different mixed function monooxygenase enzyme is a desaturase, acetylenase or a hydroxylase enzyme.
32. The recombinant polypeptide according to claims 30 or 31, wherein said polypeptide exhibits a catalytic activity which is different from the catalytic activity of either polypeptide from which it is derived.
33. A method of producing an epoxygenated fatty acid in a cell, tissue, organ or organism, said method comprising incubating a cell, tissue, organ or organism which expresses the recombinant polypeptide according to any one of claims 28 to 32 with a fatty acid substrate for a time and under conditions sufficient for at least one carbon bond of said substrate to be converted to an epoxy group.
34. The method according to claim 33, wherein the fatty acid substrate is an unsaturated fatty acid and the carbon bond of said substrate which is epoxygenated is a carbon double bond.
35. The method according to claims 33 or 34, wherein the fatty acid substrate is selected from the list comprising palmitoleic acid, oleic acid, linoleic acid, linolenic acid, 9,15-octadecadienoic acid and arachidonic acid.
36. The method according to any one of claims 33 to 35, wherein the carbon bond which

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is epoxygenated is a  $\Delta 6$  carbon bond or a  $\Delta 9$  carbon bond or a  $\Delta 12$  carbon bond or a  $\Delta 15$  carbon bond.

37. The method according to claim 36 wherein the carbon bond which is epoxygenated is a  $\Delta 12$  carbon bond.

38. The method according to any one of claims 33 to 37, wherein the epoxygenated fatty acid which is produced is vernolic acid.

39. The method according to any one of claims 33 to 38, comprising the additional first step of transforming or transfecting the cell, tissue, organ or organism with a nucleic acid molecule which encodes the recombinant epoxygenase or a homologue, analogue or derivative thereof.

40. The method according to any one of claims 33 to 39, wherein the cell, organ, tissue or organism in which the recombinant epoxygenase is expressed is derived from a bacteria, yeast, fungus, mould, insect, plant, bird or mammal.

41. The method according to claim 40 wherein the cell, organ, tissue or organism is derived from a yeast, plant, fungus or mould.

42. The method according to claim 41 wherein the yeast, plant, fungus or mould is an oleaginous yeast, plant, fungus or mould.

43. The method according to claim 42 wherein the plant is an oilseed plant which does not normally express the recombinant epoxygenase at a high level.

44. The method according to claim 43 wherein the oilseed plant is selected from the list comprising Linola<sup>®</sup> flax, oilseed rape, sunflower, safflower, soybean, linseed, sesame, cottonseed, peanut, olive or oil palm, amongst others.

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45. A plant transformed with the isolated nucleic acid molecule according to any one of claims 1 to 17 or a cell, tissue or organ derived therefrom or the progeny of said plant which also comprises said nucleic acid molecule.
46. A transformed plant which is capable of expressing the recombinant polypeptide according to any one of claims 28 to 32 or a cell, tissue or organ derived therefrom or the progeny of said plant which is also capable of expressing said recombinant polypeptide.
47. The plant or a cell, tissue or organ derived therefrom or the progeny thereof according to claims 45 or 46, comprising or derived from Linola® flax, oilseed rape, sunflower, safflower, soybean, linseed, sesame, cottonseed, peanut, olive or oil palm.
48. The plant or a cell, tissue or organ derived therefrom or the progeny thereof according to claims 45 or 46, comprising or derived from *Arabidopsis thaliana*.
49. The plant or a cell, tissue or organ derived therefrom or the progeny thereof according to claims 45 or 46, comprising or derived from *Linum usitatissimum*.
50. An antibody molecule which is capable of binding to a mixed-function epoxxygenase polypeptide or an epitope thereof.

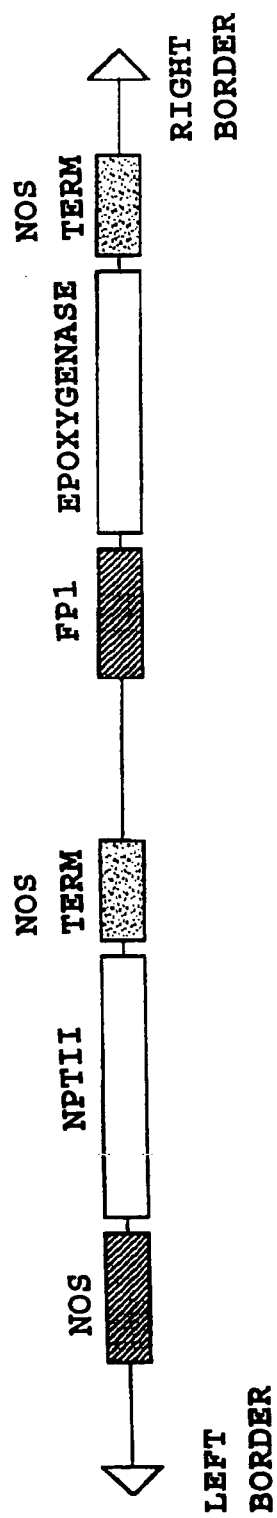


FIGURE 1

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	1			50
Cpal2	.....MGAG GR.....	..GRTSEKSV	MERVSVDPVT	FSLSELKQAI
CrepX	.....MGAG GR.....	..GRTSEKSV	MERVSVDPVT	FSLSDLKQAI
Vgal1	.....	.....	.....	.....
Crep1	.....MGGG GR.....	..GRTSQKPL	MERVSVDP.P	FTVSDLKQAI
L26296	.....MGAG GRMPV....P	TSSKKSETDT	TKRVPCCKPP	FSVGDLKKAI
X91139	.....MGAG GRMQV....S	PSPKKSETDT	LKRVPCEKPP	FTVGELKKAI
L43921	.....MGAG GRTDV....P	PANRKSEVDP	LKRVPFEKPP	FSLSQIKKAI
X92847	.....MGAG GRMSA....P	NGETEVKRN	LQKVPTSKPP	FTVGDIIKKAI
L43920	MGLAKETTMG GRGRV....A	KVEVQK.KP	LSRVPNTKPP	FTVGQLKKAI
U22378	.....MGGG GRMSTVITSN	NSEKKGGSSH	LKRAPHKPP	FTLGDILKRAI

FIGURE 2A

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	51			100
Cpal2	PPHCFQRSVI	RSSYVVQDL	IIAYIFYFLA	NTYIPTLPTS
CrepX	PPHCFQRSVI	RSSYVVQDL	IIAYIFYFLA	NTYIPNLPHP
Vgal1	.....	.....	.....	.....
Crep1	PPHCFKRSVI	RSSYIVHDA	IIAYIFYFLA	DKYIPILPAP
L26296	PPHCFKRSIP	RSFSLISDI	IIASCFYYVA	TNYFSLLPQP
X91139	PPHCFKRSIP	RSFSLIWDI	IVASCFYYVA	TTYFPLLPHP
L43921	PPHCFQRSVL	RSFSVVVDL	TIAFCLYYVA	THYFHLLPGP
X92847	PPHCFQRSLL	RSFSVVVDL	ILVSIMYYVA	NTYFHLLPSP
L43920	PPHCFQRSLL	TSFSVVVDL	SFAF.IFYIA	TTYFHLLPQP
U22378	PPHCFERSFV	RSFSVAYDV	CLSFLFYSIA	TNFFPYISSP

FIGURE 2B

	101			150	
Cpal2	FCQASVLTGL	<u>WILGHECGHH</u>	AFSNYTWFD	TVGFILHSFL	LTPYFSWKFS
CrepX	FCQASVLTGL	<u>WILGHECGHH</u>	AYSNTWVDD	TVGFILHSFL	LTPYFSWKYS
Vgal1	.....	..... <u>HH</u>	AFSDYQWIDD	TVGFILHFAL	FTPYFSWKYS
Crep1	FCQASILTGL	<u>WVIGHECGHH</u>	AFSDYQWVDD	TVGFILHSFL	MTPYFSWKYS
L26296	ACQGCVLGTGI	<u>WVIAHECGHH</u>	AFSDYQWLDD	TVGLIFHSFL	LVPYFSWKYS
X91139	ACQGVVLTGV	<u>WVIAHECGHH</u>	AFSDYQWLDD	TVGLIFHSFL	LVPYFSWKYS
L43921	AVQGCILTGV	<u>WVIAHECGHH</u>	AFSDYQLLDD	IVGLILHSAL	LVPYFSWKYS
X92847	ICQGCVCCTGI	<u>WVNAHECGHH</u>	AFSDYQWVDD	TVGLILHSAL	LVPYFSWKYS
L43920	VLQGCLLTGV	<u>WVIAHECGHH</u>	AFSKYQWVDD	VVGLTLHSTL	LVPYFSWKIS
U22378	LFQGCILTGL	<u>WVIGHECGHH</u>	AFSEYQLADD	IVGLIVHSAL	LVPYFSWKYS

FIGURE 2C

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	151		200
Cpal2	<u>HRNHH</u> SNNTSS	IDNDEVYIPK	SKSKLARIYK LLNPPGRL LLIIMFTLGF
CrepX	<u>HRNHH</u> SNNTSS	IDNDEVYIPK	SKSKLKRIYK LLNPPGRL VLVIMFTLGF
Vgal1	<u>HRNHH</u> ANTNS	LVTDEVYIPK	VKSKVKIYK ILNPPGRVF TLAFRLLIVGF
Crep1	<u>HRNHH</u> ANTNS	LDNDEVYIPK	SKAKVALYK VLNHPPGRL IMFITFTLGF
L26296	<u>HRRHH</u> SNNTGS	LERDEVFVPK	QKSAIKWYK YLNPLGRIM MLTVQFVLGW
X91139	<u>HRRHH</u> SNNTGS	LERDEVFVPK	KKSDIKWYK YLNPLGRTV MLTVQFTLGW
L43921	<u>HRRHH</u> SNNTGS	LERDEVFVPK	QKSCIKWYK YLNPPGRVL TLAVTLLTGW
X92847	<u>HRRHH</u> SNNTGS	LERDEVFVPK	PKSQLGWYK YLNPPGRVL SLTITLLTGW
L43920	<u>HRRHH</u> SNNTGS	LDRDEVFVPK	PKSKVAWFSK YLNPLGRAV SLLVTLLTIGW
U22378	<u>HRRHH</u> SNIGS	LERDEVFVPK	SKSKISWYK YSNPPGRVL TLAATLLLGW

FIGURE 2D

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	201			250
Cpal2	PLYLLTNISG	KKY.DRFANH	FDPMSPIFKE	RERFQVFLSD LGLLAVFYGI
CrepX	PLYLLTNISG	KKY.DRFANH	FDPMSPIFKE	RERFQVFLSD LGLLAVFYGI
Vgal1	PLYLFTNVSG	KKY.ERFANH	FDPMSPIFTE	REHVQVLLSD FGLIAYAVVV
Crep1	PLYLFTNVSG	KKY.ERFANH	FDPMSPIFKE	RERFQVLLSD LGLLAVLYGV
L26296	PLYLAFNVSG	RPY.DGFACH	FFPNAPIYND	RERLQIYLSG AGILAVCFGL
X91139	PLYWAFNVSG	RPYPEGFACH	FHPNAPIYND	RERLQIYVSD AGILAVCYGL
L43921	PLYLALNVSG	RPY.DRFACH	YDPYGPYISD	RERLQIYISD AGVLAVVYGL
X92847	PLYLAFNVSG	RPY.DRFACH	YDPYGPYINN	RERLQIFISD AGVLGVCYLL
L43920	PMYLAFNVSG	RPY.DSFASH	YHPYAPIYSN	RERLLIYVSD VALFSVTYSL
U22378	PLYLAFNVSG	RPY.DRFACH	YDPYGPYIFSE	RERLQIYIAD LGIFATTFVL

FIGURE 2E

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251	300
Cpal12	KVAVANKGAA WVACMYGVPV LGVFTFFDVI TFLHHTHQSS PHYDSTEWNW
CrepX	KVAVANKGAA WVACMYGVPV LGVFTFFDVI TFLHHTHQSS PHYDSTEWNW
Vgal1	RQAVLAKGGA WVMCIYGVVPV LAVNAFFVLI TYLHHTHLSL PHYDSSSEWDW
Crep1	KLAVAAKGAA WVTICIYGIPV LGVFIFFDII TYLHHTHLSL PHYDSSSEWNW
L26296	YRYAAAQGMA SMICLYGVPL LIVNAFLVLI TYLQHTHPSL PHYDSSSEWDW
X91139	YRYAAAQGVA SMVCLYGVPL LIVNAFLVLI TYLQHTHPSL PHYDSSSEWDW
L43921	FRLAMAKGLA WVVCVYGVPL LVVNGFLVLI TFLQHTHPAL PHYTSSEWDW
X92847	YRIALVKGLA WLLCVYGVPL LVVNGFLVLI TYLQHTHPSL PHYDSTEWDW
L43920	YRVATLKGLV WLLCVYGVPL LVVNGFLVTI TYLQHTHFAL PHYDSSSEWDW
U22378	YQATMAKGLA WVMRIYGVPL LIVNCFVMI TYLQHTHPAI PRYGSSEWDW

FIGURE 2F

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Cp12	IRGALSAIDR	DFGFLNSV FH	DVTHTHVMHH	LFSYIPHYHA	KEARDAIKPI
CrepX	IRGALSAIDR	DFGFLNSV FH	DVTHTHVMHH	LFSYIPHYHA	KEARDAIKPI
Vgal1	LR.....	.....	.....	.....	.....
Crep1	LRGALSTIDR	DFGFLNSVLH	DVTHTHVMHH	LFSYIPHYHA	KEARDAINTV
L26296	LRGALATVDR	DYGILNKV FH	NITDTHVAHH	LFSTMPHYNA	MEATKAIKPI
X91139	LRGALATVDR	DYGILNKV FH	NITDTHVAHH	LFSTMPHYHA	MEVTKAIKPI
L43921	LRGALATVDR	DYGILNKV FH	NITDTHVAHH	LFSTMPHYHA	MEATKAIKPI
X92847	LRGALATCDR	DYGVNLKV FH	NITDTHVVHH	LFSTMPHYNA	MEATKAVKPL
L43920	LKGALATMDR	DYGILNKV FH	HITDTHVAHH	LFSTMPHYHA	MEATNAIKPI
U22378	LRGAMVTVDR	DYGVNLKV FH	NIADTHVAHH	LFATVPHYHA	MEATKAIKPI

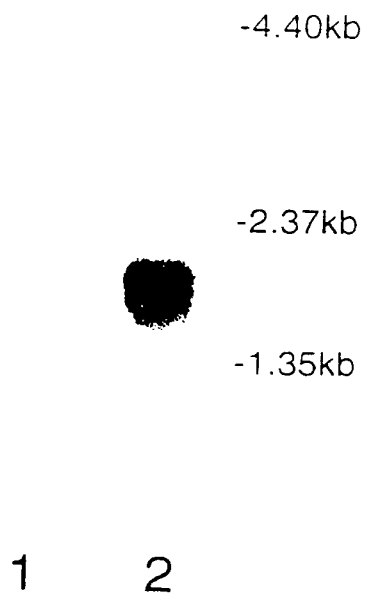
FIGURE 2G

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	351					394
Cpal2	LGDFYMDRT	PILKAMWREG	RECMYIEPDS	..KLKGVYWY	.HKL	
CrepX	LGDFYMDRT	PILKAMWREG	RECMYIEPDS	..KLKGVYWY	.HKL	
Vgal1	.....	.....	.....	.....	.....	
Crep1	LGDFYKIDRT	PILKAMWREA	KECIFIEPEK	GRESKGVYWY	.NKF	
L26296	LGDIYQFDGT	PWYVAMYREA	KECIYVEPDR	EGDKKGVYWY	NNKL	
X91139	LGDIYQFDGT	PWVKAMWREA	KECIYVEPDR	QGEKKGVFWY	NNKL	
L43921	LGEYRFEDET	PFVKAMWREA	RECIYVEPDQ	STESKGVFWY	NNKL	
X92847	LGDIYQFDGT	PIYKEMWREA	KECLYVEKDE	SSQKGVFWY	KNKL	
L43920	LGEYQFDDT	PFYKALWREA	RECLYVEPDE	GTSEKGVYWY	RNKY	
U22378	MGEYRYDGT	PFYKALWREA	KECLFVEPDE	GAPTQGVFWY	RNKY	

FIGURE 2H

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**FIGURE 3**



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A



1

2



3

4

B



1

2

3

**FIGURE 5**

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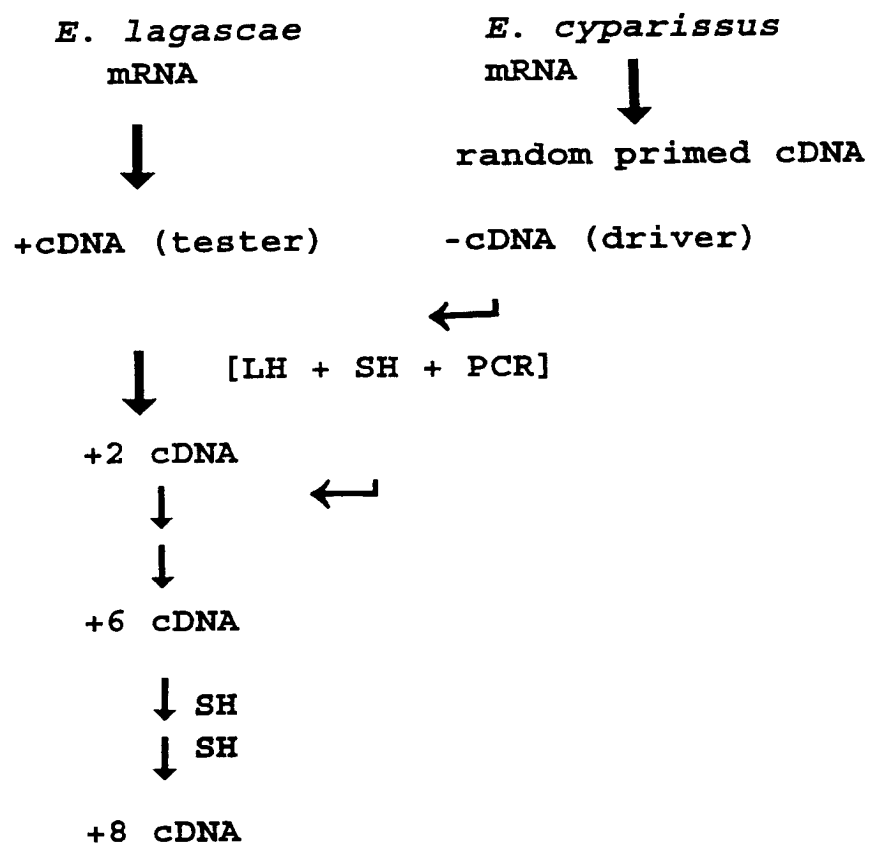
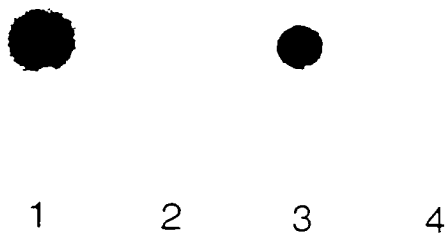


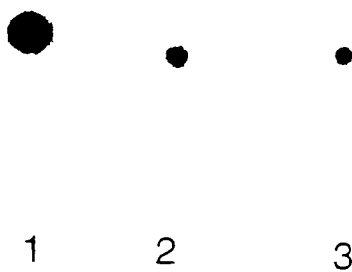
FIGURE 6

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A

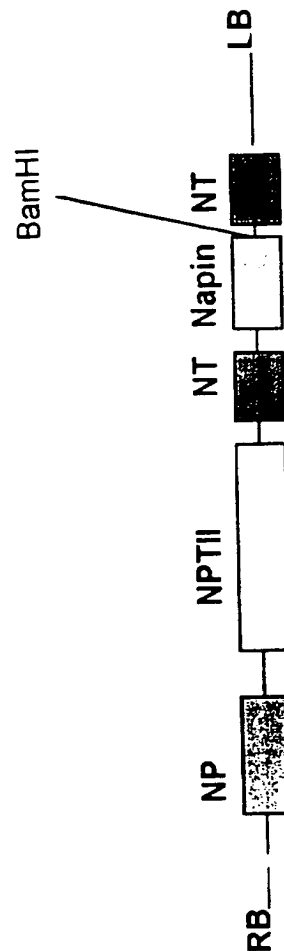


B



**FIGURE 7**

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**FIGURE 8**

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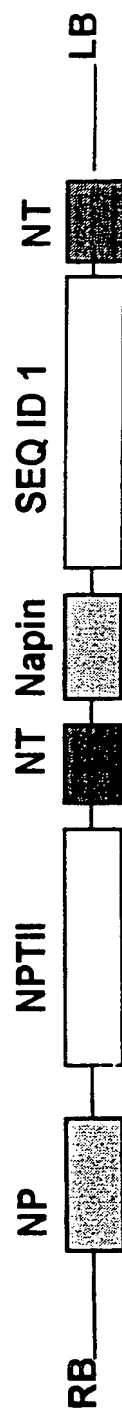


FIGURE 9

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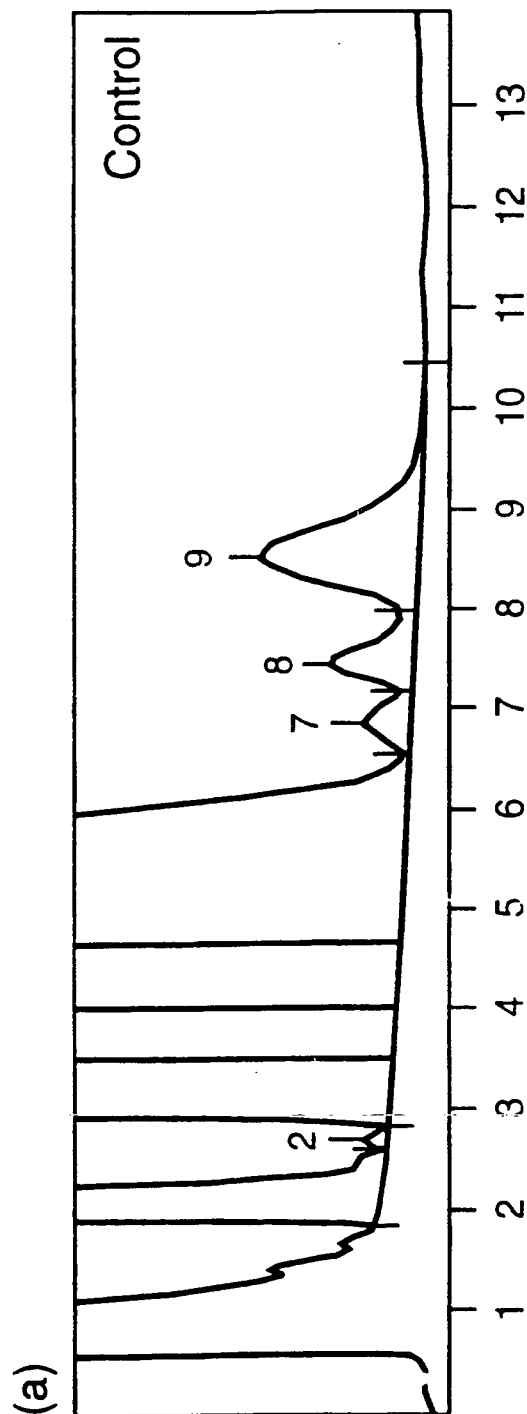


FIGURE 10A

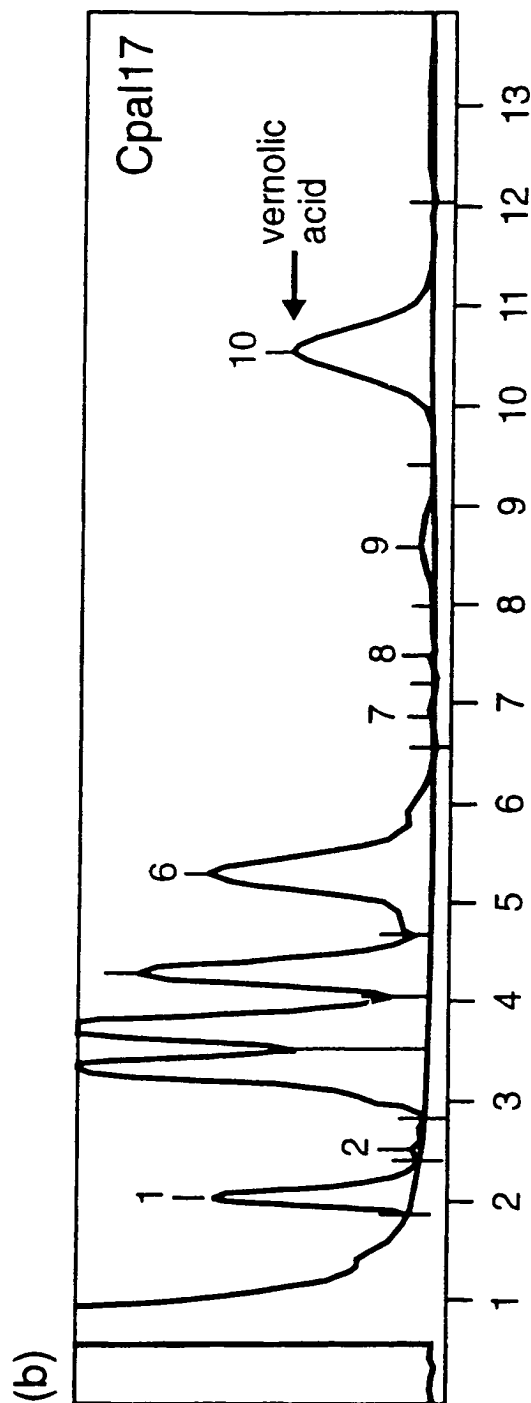


FIGURE 10B

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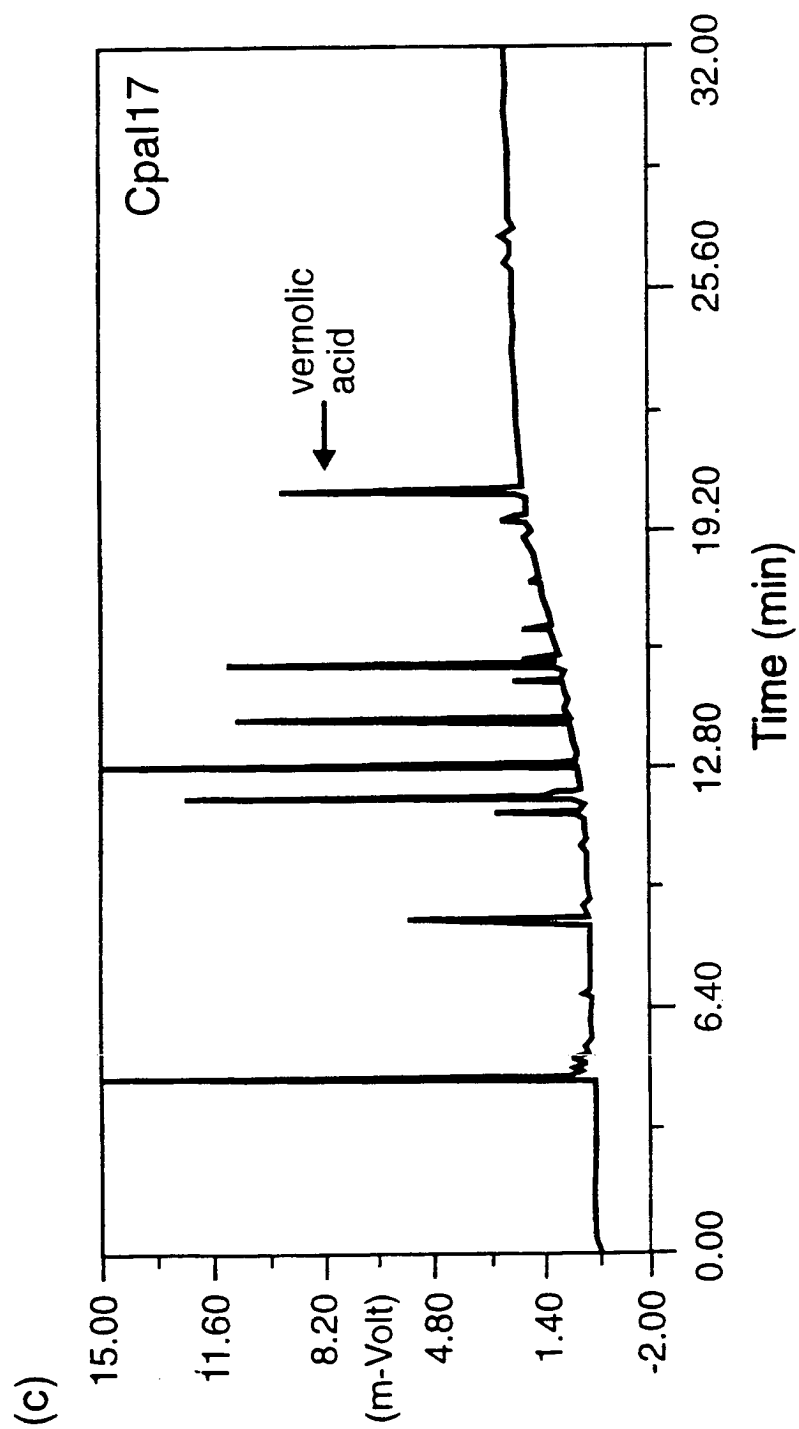


FIGURE 10C

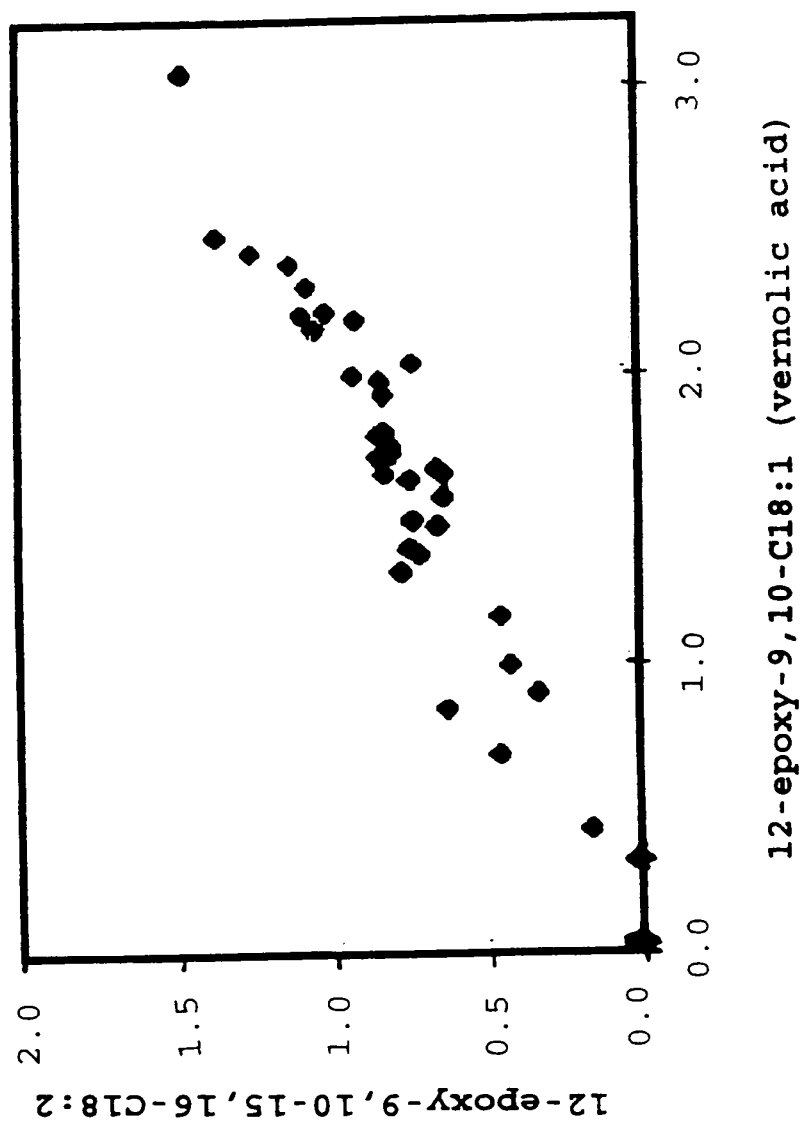


FIGURE 11

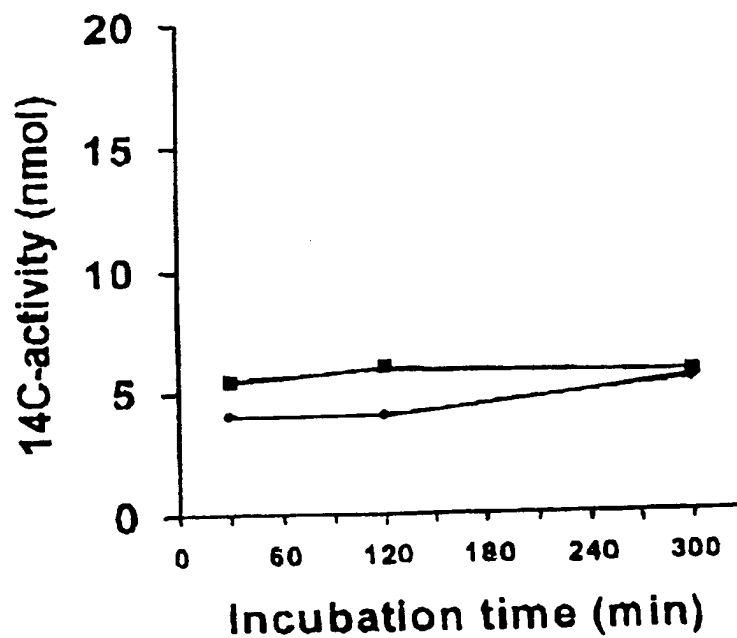


FIGURE 12

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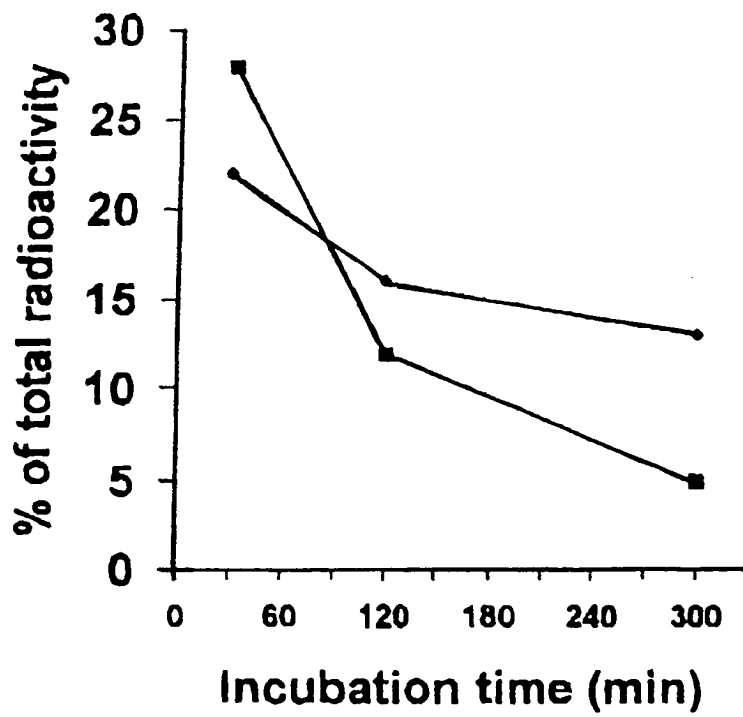


FIGURE 13

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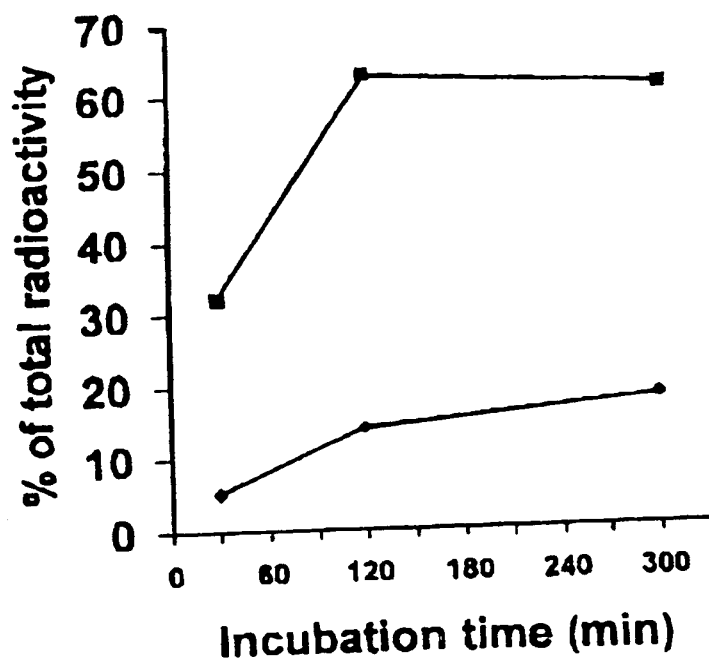


FIGURE 14

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 98/00246

## A. CLASSIFICATION OF SUBJECT MATTER

Int Cl<sup>6</sup>: C12N 15/53, 9/02

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
DERWENT DATABASE-WPAT, CHEMICAL ABSTRACTS.-Keywords below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
DERWENT DATABASE-USPM; SWISS-PROT, PIR, EMBL, DGENE- Sequence Search; MEDLINE - Keywords

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
DERWENT DATABASE (WPAT, USPM) - Keywords: EPOX:, C12N-9/IC C12N-015/IC; CHEM. ABSTRACTS, MEDLINE-Keywords: epoxidase, epoxygenase, gene; DGENE SWISS-PROT, PIR, EMBL- Seq. ID.NOS: 1-6, 19, 20

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96/10074 (VANDERBILT UNIVERSITY) publ. 4 April 1996, (see Examples and claims)	1-50

☒ Further documents are listed in the continuation of Box C

☐ See patent family annex

* Special categories of cited documents:	
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"E" earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search  
15 June 1998

Date of mailing of the international search report  
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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 98/00246

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Methods in Enzymology, Vol. 187, 1990, J.H. Capdevilla <u>et al.</u> , "Cytochrome P-450 Arachidonate Oxygenase" pp. 385-394 (see entire document)	1-50
Y	Comparative Biochemistry and Physiology, Vol. 83C, No. 1, 1986, M.F. Christian and S. J. Yu, "Cytochrome P-450-Dependent Monooxygenase Activity in the Velvetbean Caterpillar, <i>Anticarsia Gemmatilis</i> Hubner" pp. 23-27 (see entire document)	1-50
Y	Advances in Prostaglandin, Thromboxane and Leukotriene Research, Vol. 21, 1991, M.F. Romero <u>et al.</u> , "An Epoxygenase Metabolite of Arachidonic Acid 5,6 Epoxy-Eicosatrienoic Acid Mediates Angiotensin-induced Natriuresis in Proximal Tubular Epithelium" pp. 205-208 (see entire document)	1-50
Y	Drug Metabolism and Disposition, Vol. 24, No. 6, June 1996, R. M. Laethem <u>et al.</u> , "Epoxidation of C <sub>18</sub> Unsaturated Fatty Acids by Cytochromes P450C2 and P450CAA" pp. 664-668 (see entire document)	1-50
Y	Archives of Biochemistry and Biophysics, Vol. 303, No. 1, May 15, 1993, M. Bafor <u>et al.</u> , "Biosynthesis of Vernoleate 9 <i>cis</i> -12-Epoxyoctadeca- <i>cis</i> -9-enoate) in Microsomal Preparations from Developing Endosperm of <i>Euphorbia lagascae</i> ", pp. 145-151 (see entire document)	1-50
P,X	Science, Vol. 280, 8 May 1998, M. Lee <u>et al.</u> , "Identification of Non-Heme Diiron Proteins that Catalyze Triple Bond and Epoxy Group Formation" pp. 915-918 (see p. 916)	1-50